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THE BIOCHEMICAL BASIS OF TEMPERATURE-SENSITIVITY IN *MICROCOCCUS CRYOPHILUS*

by

NEIL L. MALCOLM

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Biochemical Basis of Temperature-Sensitivity in Micrococcus cryophilus" submitted by Neil L. Malcolm in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Micrococcus cryophilus, an obligate psychrophile, has been investigated with a view to explaining the biochemical basis of its temperature-sensitivity. A minimal medium, capable of supporting the growth of this organism throughout its temperature range, -4°C to 25°C, was devised. A study was made of certain aspects of the cellular chemistry of the psychrophile in cultures maintained at 14°C as compared with that in cultures transferred from 14°C to 30°C. In certain cases the psychrophile was compared with a mesophilic mutant derived from it. This mutant is capable of growth at 30°C.

No specific supplementation of the growth medium was found to sustain growth of the psychrophile at 30°C. Studies pertaining to the cellular integrity of the organism during incubation at 30°C indicated that permeability damage occurred subsequent to the death of the cell and did not appear to be the cause of death. Intermediary metabolism, respiration, oxidative phosphorylation were likewise ruled out as possible sites of exceptionally labile enzymes, the processes examined exhibiting similar, if not greater, rates of activity at 30°C as at 14°C.

Both amino acids and adenosine-5'-triphosphate were found to accumulate in the micrococcus at 30°C, suggesting an inhibition of macromolecular synthesis. Colorimetric analyses and incorporation studies, using radioactive analogues of leucine and uracil, both indicated that first protein

synthesis, then ribonucleic acid synthesis halted very soon after transfer to 30°C. The cessation of protein synthesis coincided with the loss of viability of the cell. An assay of the activities of the amino acid-transfer ribonucleic acid synthetases revealed six with reduced activities at the higher temperature.

Studies of the turnover and degradation of ribonucleates at 30°C indicated an increased rate of breakdown whether determined by the total ribonuclease activity of cell extracts or by the release from the cell of quantities of 5'-nucleotides of the four main ribonucleic acid bases.

A final series of experiments utilizing the known inhibiting properties of chloramphenicol showed that leucine incorporation could be induced to continue at an increased rate for at least twice as long at 30°C if the intracellular level of ribonucleic acid were first increased. A possible relationship between this finding, the presence of increased ribonuclease activity and the reduced activities of certain amino acid-transfer ribonucleic acid synthetases is discussed.

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INTRODUCTION

Two properties distinguish obligately psychrophilic micro-organisms from mesophilic micro-organisms: the ability to grow and reproduce at 0°C and the inability to grow at moderate temperatures of 25°C to 30°C. This investigation is concerned only with the latter phenomenon and seeks to find the biochemical basis or bases determining the low maximum growth temperature of an obligate psychrophile, Micrococcus cryophilus.

Little is known about the biochemical basis of limitation of growth at the upper temperature limit of any micro-organism, or indeed if a similar biochemical basis is involved in most cases. Consequently, there are few guide-lines for an investigation into extreme temperature-sensitivity. However, with a foreknowledge of the effects of increased temperature on the structure and function of chemical constituents normally found in the cell, several areas may be postulated as possible sites of temperature-induced lability. These include proteins (Edwards and Rettger, 1937), DNA (Marmur and Doty, 1959) and RNA (Califano, 1952; Strange and Shon, 1964), and the liquid-crystalline nature of membrane lipids (Luzzati and Husson, 1962; Byrne and Chapman, 1964).

Loss of catalytic activity by proteins is usually assumed to be a major factor, and Edwards and Rettger (1937) reported excellent agreement between the maximum temperature for growth of several bacilli and the temperatures at

which the respiratory enzymes, catalase, indophenol oxidase and succinate dehydrogenase were inactivated. A study by Hagen and Rose (1962) with a psychrophilic strain of cryptococcus showed that the maximum temperature for growth of this yeast was probably determined by the heat inactivation of one or more respiratory enzymes. Upadhyay and Stokes (1963) have reported the presence of a heat-sensitive formate hydrogenlyase in a psychrophilic bacterium, while Burton and Morita (1965) showed that the malate dehydrogenase in a psychrophilic marine bacterium was abnormally sensitive to heat denaturation, although in neither of these reports was there evidence to show that the heat sensitivity of the enzyme determined the maximum temperature for growth of the organism. Evison and Rose (1965), in a comparative study on factors affecting the upper temperature growth limit in psychrophilic strains of Arthrobacter, Candida and Corynebacterium erythrogenes which have maximum temperatures ranging from about 22°C (Candida) to 34°C (C. erythrogenes), noted the respiratory activities of the Arthrobacter and the Candida were adversely affected after the transfer of growing cultures from the optimum to a supermaximum temperature. When cell-free extracts of these two organisms were assayed for the activities of tricarboxylic acid cycle enzymes, certain activities were found to be lower in extracts exposed to higher temperature, but no one enzyme appeared to be particularly susceptible to heat inactivation. The authors

concluded that loss of enzyme activity and possibly also inactivation of enzyme synthesis were major factors in determining the maximum temperatures for growth. All of these reports would indicate that the maximum temperatures for growth, whether high or low, are possibly determined, to some extent at least by the heat denaturation of respiratory enzymes.

Denaturation and degradation of DNA and RNA as factors in determining the maximum temperature for growth have not been widely reported in the literature, although much work has been done on the relationship of structure to function in these macromolecules and the effect of temperature on this relationship. Evison and Rose (1965) did mention that no evidence could be found for the degradation of RNA at higher temperatures in the organisms studied. Pace and Campbell (1966) searching for a correlation between the maximal growth temperature of a range of organisms and the thermal stability of the cellular components, noted that the denaturation temperatures (Tm) of the 70S ribosomal fractions did vary with the maximum growth temperatures of the cells. However the Tm for the ribosomes of psychrophilic cells with a maximum growth temperature of 20°C was 68°C indicating that one had no bearing to the other. Marmur (1961) has shown for a series of different mesophilic genera that DNA lability, as determined by Tm values, was unrelated to maximum growth temperature.

Changes in lipid constitution have been examined as factors limiting growth at elevated temperature. Kates and Hagen (1964) studying a species of Serratia found that as the incubation temperature was increased so was the degree of saturation of fatty acids. Marr and Ingraham (1962) reported a similar situation in Escherichia coli grown in complex and minimal media. Growth at a particular temperature, however, did not result in a unique fatty acid composition, since altering the nutrition independently of temperature also resulted in major changes in fatty acid composition. They concluded that fairly wide differences in fatty acid composition have no effect on growth rate and these differences appear to have little physiological effect. Kates and Baxter (1962) comparing the total extractable lipid in mesophilic and psychrophilic strains of Candida, found it to be approximately equal in each case, but that of the psychrophile contained a higher percentage of unsaturated fatty acids. If elevated temperature were to affect cellular lipids in such a way that growth halted, probably the membrane of the cell with its lipoprotein structure might be the most obvious structure of the cell to look for damage. Gross changes in membrane function would perhaps be revealed as a loss of control over cellular permeability. Haight and Morita (1966) have observed leakage of cellular components into the surrounding menstruum when Vibrio marinus was subjected to temperature above the organism's maximum growth temperature. Whereas Hagen et al. (1964),

in a similar experiment with a different psychrophile, indicated that loss of viability occurred prior to lysis, these authors have not established this sequence of events and it remains doubtful whether this factor is the primary cause of cell death.

The denaturation of enzymes by heat has been and continues to be the main area of investigation in studies on temperature-sensitivity and the maximum growth temperatures of micro-organisms. The biological activity of proteins depends upon the three-dimensional configuration of their constituent polypeptide chains. It is believe that, in the intracellular milieu, one of the many possible configurations for a polypeptide with a given sequence of amino acids is more stable than any of the others. This "native" configuration is maintained by intramolecular bonds, many of which are noncovalent and individually weak. By participating in these bonds, or by permitting them to form, each amino acid in the molecule probably contributes to some extent in the activity of an enzyme.

The forces holding a protein in its native configuration usually do so only over a narrow temperature range. Upon heating, most proteins in solution undergo denaturation with attendant loss of catalytic activity. When a certain temperature range is reached, the rate of thermal inactivation of most enzymes increases with a small rise in temperature (Haurowitz, 1963). Furthermore, the lower range of temperatures at which proteins are inactivated overlaps the upper range at which biological systems operate. Thus many

proteins are known to become inactivated between 40°C and 60°C, and the temperature versus growth rate curve quoted for Escherichia coli by Ng et al. (1962) suggests that thermal inactivation of enzymes may become a biologically significant event for these cells as low as 35°C.

Taken together, these facts suggest that single mutational alterations in the amino acid sequence of a protein may in some cases lower the temperature at which denaturation occurs. This expectation is borne out by several instances of heat-sensitizing alterations in microbial proteins (Maas and Davis, 1952; Horowitz and Fling, 1953). Of late such mutational alterations, some induced, some discovered fortuitously, have been sought by workers wishing to use temperature-sensitive mutants in the study of protein and nucleic acid synthesis and their regulation in bacterial cells. There is no evidence against the notion that many cellular proteins might have this temperature of inactivation lowered by a single mutational step that does not seriously hinder normal catalytic activity at lower temperatures. The work being carried out using 'conditionally lethal' mutants of this type obviously has relevance to the present study.

Edgar and his associates (Epstein et al. 1963) have taken advantage of temperature-sensitizing mutations to isolate conditionally lethal mutants of coliphage T4. Their strains are able to multiply in host cells at 25°C but not at 42°C, while the parental phage can multiply at either

temperature. Over 300 such mutants have been isolated, and genetic mapping places them in about thirty-five cistrons scattered over the T4 genome. The results of complementation tests are consistent with the idea that these strains arose by mis-sense mutations substituting "incorrect" amino acids for the "correct" ones in polypeptides, and permitting synthesis of an altered protein functionally defective only at the higher temperature. The large number of these isolated reinforces the conclusion that many if not all proteins can be altered in a similar way.

Horiuchi et al. (1961) have isolated a mutant of E. coli in which the regulation of beta-galactosidase synthesis has become temperature-sensitive. This strain behaves as if the aporepressor, (the produce of the regulatory gene, i) for the lac operon is inactivated above 40°C. Recently, Sadler and Novick (1965) have presented information on a mutant in which the actual formation of the aporepressor appears to be temperature-sensitive. Gallant (1962) and Gallant and Stapleton (1963) have studied a mutant of E. coli in which the synthesis of an aporepressor for alkaline phosphatase has become temperature-sensitive.

Two other groups of workers are currently seeking temperature-sensitive mutants blocked at higher temperatures in the synthesis of macromolecules. Neidhardt and his coworkers are investigating the synthesis of RNA and its regulation using such mutants. Early reports (Eidlic and Neidhardt, 1965) have indicated that in some cases a specific

amino acid - tRNA synthetase has become heat-labile. Jacob and his group are seeking temperature-sensitive mutants with DNA-synthesizing systems nonfunctional at around 40°C. To date, only the isolation and characterization of such mutants has been described (Kohiyama et al. 1966).

These observations of many different mutations rendering cells temperature-sensitive in varying cellular functions substantiates the thesis that most proteins can be altered in this way without halting catalytic activity completely. Such single mutations are desired and selected for the purposes of specific study, however, and it should be recognized that temperature-sensitivity in an obligate psychrophile probably devolves down to more than a single heat-sensitizing mutation otherwise a mutation to mesophily should quickly occur under favourable conditions.

The organism studied in this investigation was first isolated in 1950 and taxonomically described by McLean et al. (1951). The organism is an unusually large coccus occurring in chains, clusters and diploforms, and as single spheroid cells. These cells, under optimum conditions, vary in size, averaging 1.6 microns in diameter, occasional small cells measuring 1.0 micron and large cells measuring 3.6 microns in diameter. They are non-motile (McLean, 1964) and aerobic. Gram stains show Gram-positive cells with a predominant number of Gram-negative cells irrespective of age. Gelatin is not liquefied, indole and H_2S are not formed, and nitrates are not reduced. The organism produces

no haemolysis on blood agar. It is not capable of growth in sodium citrate or in $NH_4H_2PO_4$ as sole sources of carbon and nitrogen respectively. There is no fermentation of xylose, glucose, lactose, sucrose, maltose, cellulose, mannitol, dulcitol or salicin. Urease and catalase are formed but no oxidase. Growth occurs at -4°C and up to a maximum of 25°C. Growth is best at pH 7.2 but occurs in the range 5.5 to 9.5.

On the basis of the above findings, McLean et al. classified the organism as a micrococcus and suggested the name Micrococcus cryophilus since it grew only in a low temperature range.

DeLamater and Woodburn (1952) finding that the size of this cell made it suitable for cytologic study, observed nuclear bodies varying in size and position within the cell and suggested that these represented various stages of a mitotic process, a suggestion which came under strong criticism from Bisset (1954) who showed that what was considered to be a "metaphase spindle" was in fact a septum originating between two cells. Later DeLamater (1962) withdrew his conclusions about mitosis in M. cryophilus.

Recently the taxonomic position of this organism has been investigated by Martinec and his colleagues (Mazanec et al. 1966; Bohacek et al. 1966). Studies of ultrathin sections of M. cryophilus revealed that in morphological structure and cell groupings after division it differed from other Gram-positive cocci. It resembled pediococci,

especially in its cell division and cell grouping and a recommendation was made that it should be discarded from the genus Micrococcus. The GC content in DNA of M. cryophilus (41.3%), too, has shown a marked difference from micrococci which have a much higher GC content (65.6 - 75.5%). Again, this resembles pediococci, especially Pediococcus cerevisiae which has 44.4% GC.

Apart from these investigations, no work has been carried out on this organism with a view to gaining more information about its biochemistry or especially concerning the most interesting aspect of its physiology, its inability to grow above 25°C.

MATERIALS AND METHODS

I. GENERAL

Test Organisms

Micrococcus cryophilus ATCC 15174 (American Type Culture Collection) and a mutant with a higher maximum growth temperature were used throughout the course of this experiment. These two strains will be referred to as ATCC 15174 and 15174-RB respectively.

Stock cultures were maintained on trypticase soy agar (Baltimore Biological Laboratories) at 0°C.

Growth Conditions

Cultures were grown in baffled flasks in a New Brunswick Psychrotherm incubator shaker (New Brunswick Scientific Co., New Jersey, U.S.A.). Flasks of 300 ml. containing 100 ml. medium were shaken at 300 revolutions per minute.

Media

Three media were used for the cultivation of the test organisms:

1) a complex medium, trypticase soy broth (TSB)

2) a minimal medium, glutamate-salts-medium (GSM) with the following composition: buffered salts medium (SM)-

KH_2PO_4	5 g.	$MnCl_2.4H_2O$	0.01 g.
Sodium citrate	5 g.	$FeCl_3$.	0.001 g.
NaCl	5 g.	Distilled water	1 litre
$MgSO_4.7H_2O$	0.08 g.		

The pH of this medium was adjusted to 7.2 with KOH.

This base was sterilized in the autoclave and supplemented, just prior to use, with 0.05 M L-glutamic acid which was prepared separately and sterilized by filtration.

When necessary, 1.5% Difco agar was added to obtain a solid medium (TSA, GSA).

3) a semi-defined medium utilized in a study on the basic nutrient requirements of the psychrophile and its mutant. This medium contained the buffered salts base described under 2) with the addition of glucose (1%) or any other carbohydrate source, Difco vitamin-free Casamino Acids (1%) and Difco Yeast Extract (0.02%) as the main carbon, nitrogen and vitamin sources respectively (all w/v).

Determination of the Basic Nutrient Requirements of ATCC 15174

The semi-defined medium described under media 3) was employed in the determination of the basic nutrient requirements of ATCC 15174. The procedure consisted of removing from the medium either glucose, Casamino Acids or Yeast Extract in turn and substituting respectively, another carbon source, or more specific nitrogen and purine, pyrimidine and vitamin supplements. These specific substitutions were made either as single compounds or as mixtures of compounds. Acid-washed glassware was used throughout these determinations.

Replacement of Casein Hydrolysate

Amino acid solutions: the L-form of each of the 18 amino acids (Calbiochem., Los Angeles, U.S.A.) was dissolved individually in 50 ml. distilled water to a concentration of 3% (w/v).

Complete amino acid mixture: 1 ml. of each of the individual amino acid solutions were combined, the pH adjusted to 7.0, and the total volume brought to 20 ml. with distilled water.

Solutions of amino acid groups: 5 ml. amounts of the individual amino acid solutions listed in the following groups were mixed. The pH of each mixture was brought to 7.0 and the total volume adjusted to 25 ml. with distilled water.

a) Arginine, cysteine, cystine, methionine

b) Isoleucine, leucine, valine

c) Phenylalanine, tryptophan, tyrosine

d) Aspartic acid, glutamic acid, histidine, threonine

e) Alanine, glycine, proline, serine

Five ml. amounts of the individual amino acids, the complete amino acid mixture or the solutions of amino acid groups were sterilized by filtration.

Three per cent solutions of the inorganic salts $(NH_4)_2SO_4$ and $NaNO_3$ were also prepared and sterilized. These were added to a suitable medium to determine whether inorganic compounds would suffice as the sole source of nitrogen for this organism.

The prepared solutions were added to the base at a ratio of 1:19 (v/v).

Purine and Pyrimidine Requirements

Yeast extract in the medium was replaced by the addition of purines and pyrimidines and also vitamins. Initially the first two were included in a medium where the level of yeast hydrolysate had been reduced to 0.025% (w/v) concentration.

Purines (Calbiochem): adenine, guanine, xanthine, hypoxanthine.

Pyrimidines (Calbiochem): uracil, cytosine, 5-methylcytosine, thymine.

Solutions of 0.4% (w/v) of these bases in distilled water were prepared using alkali where necessary to obtain solution.

Purine mixture: equal volumes of adenine, guanine, xanthine and hypoxanthine were combined.

Pyrimidine mixture: equal volumes of uracil, cytosine, 5-methylcytosine and thymine were combined.

These solutions were sterilized by filtration and added to the base at a ratio of 2:25 (v/v).

Vitamin Requirements

Components required for growth as ascertained in the previous section were assembled, yeast extract being omitted.

Vitamins (General Biochemicals, Inc., Ohio, U.S.A.):

nicotinic acid
DL-pantothenate, calcium salt
folic acid
pyridoxal phosphate-hydrochloride
riboflavin
thiamine-hydrochloride
biotin
p-amino benzoic acid
inositol

The first six vitamins were made in 0.006% (w/v) concentrations in distilled water, the last three at 0.005% concentrations.

Carbohydrate Requirements

Throughout the earlier tests glucose, at a final concentration of 1% (w/v) had been employed as the source of carbon in the medium. This was now replaced and compared individually with a series of carbohydrates.

D-sugars (Sigma Chemical Co., St. Louis, Mo.):

glucose	mannitol	arabinose	adonitol
sucrose	xylose	galactose	mannose
sorbitol	maltose	raffinose	sorbose
lactose	rhamnose	laevulose	starch
salicin	dulcitol	trehalose	
melibiose	melizitose	inulin	

Intermediates (Nutritional Biochemical Corporation):

pyruvate, sodium salt

acetate, sodium salt

alpha-ketoglutarate

malate, sodium salt

The sugars were prepared in 10% solutions (w/v) in distilled water, the intermediates in 5% (w/v) solutions. Both were sterilized by filtration and added to the base in the ratio 1:19 (v/v).

Glucose Determination

It became necessary during these tests to study the concentration of glucose present in the medium at different stages of growth. This was done by examining an aliquot of cell-free supernatant, obtained by centrifuging down the cells at 25,000 g. A dilution of this fraction was assayed for D-glucose present by first catalyzing its phosphorylation by hexokinase in the presence of adenosine triphosphate (ATP). The glucose-6-phosphate formed was then oxidized in the presence of nicotinamide adenine dinucleotide (NAD^+) by glucose-6-phosphate dehydrogenase, the reduced nicotinamide adenine dinucleotide ($NADH.H^+$) formed being determined spectrophotometrically at 340 millimicrons in a Beckman DU Spectrophotometer. This served as a measure of the glucose-6-phosphate formed initially from glucose (Stein, 1963). A cuvette was charged with triethanolamine buffer (750 millimoles; pH 7.5), $MgSO_4$ (0.25 millimole) NAD^+(1.2 millimole), ATP (1.6 millimole) and water to a volume of 2.8 ml.

A 0.2 ml. quantity of sample, or distilled water in the case of the blank, was added and the reaction started by the addition of 0.2 mg. hexokinase (Boehringer) plus 0.4 mg. glucose-6-phosphate dehydrogenase (Boehringer) in 0.02 ml. The optical density was read after 10 minutes and the readings compared with those obtained using known concentrations of glucose.

Measurement of Growth Response

Two tests were used: either, the supplemented base was made up in liquid form, the inoculum added, and growth at 14°C followed turbidimetrically in a Bausch and Lomb Spectronic 20 at wavelength 600 millimicrons; or, the medium minus its defined supplement was solidified by the addition of 1.5% agar, melted and poured into a 7-inch Petri dish along with an inoculum and allowed to set and cool to the temperature of inoculation. Sterile assay cylinders were then placed on the surface of the agar and 0.2 ml. of the prepared solution of the specific growth factor lacking in the medium added to each. In this case growth response was followed visually after 120 hours incubation at 14°C by estimating and comparing the zones of growth or inhibition around the individual cups.

The inoculum was prepared by growing the cells in the semi-defined medium for 48 hours then harvesting at 25,000 g. in a refrigerated centrifuge, washing the pellet three times in sterile 0.05 M phosphate buffer, pH 7.0, at 0°C and then resuspending the cells to a known density

in buffer. In the liquid medium 1% by volume of a cell suspension of $O.D._{600}$ 0.1 was added whereas, on plating, 5 ml. of cells at a density of 0.4 were added.

Measurement of Growth

Three parameters were employed to evaluate the growth of ATCC 15174: viable cell count, turbidity and dry weight.

A 1% inoculum, by volume, prepared from cells grown at 14° in G.S.M. and subcultured four times after 18 hours incubation, was added to fresh medium at the same temperature. Samples were removed as desired and examined as follows:

1. Viable Cell Counts: dilutions were made in 0.1% peptone water at 4°C. Ten 0.01 ml. samples of a suitable dilution were plated on to the surface of a plate poured 24 hours previously and allowed to dry at 14°C. Surface plates were used to avoid possible heat-damage to the cells by mixing them with molten agar. An incubation time of 96 hours at 14° was sufficient to allow colonial numbers to be counted easily. This procedure was used in all the viability studies.
2. Turbidity: the optical density of the culture at 600 millimicrons was compared with a blank of unincubated medium in a Bausch and Lomb Spectronic 20.
3. Dry Weight: a 10 ml. sample of the culture was harvested by centrifugation at 0°C, and resuspended in

5 ml. 0.05 M phosphate buffer, pH 7.0. A 4 ml. quantity of this suspension was transferred to a dried aluminum-foil cup of known weight and the whole unit placed in a drying oven at 90°C for 24 hours. (In the case of the zero time tubes, 8 ml. samples were withdrawn and dried). The dried material was cooled to room temperature in a dessicator, weighed, and the dry weight/ml. of original culture established. The average of three samples was recorded and used in the preparation of a growth curve showing dry weight of cells versus the age of the culture.

This organism was studied under defined and consistent conditions of cultivation and consequently it was desirable to relate dry weight to turbidity measurements. A series of bacterial suspensions were prepared and the turbidity of each determined. Samples of the undiluted suspension were dried, the dry weight noted, and the results plotted graphically against the absorbance readings.

When a comparison of growth rates of logarithmic phase cultures under differing circumstances became necessary this was expressed as the value of k which is given by the relationship:

$$k = \frac{\ln 2}{\text{mass doubling time in hours}}$$

Viability Studies

The technique used to study the effect of exposure to moderate temperature, between 25°C and 30°C, on cells of ATCC 15174 was a transfer technique whereby cells grown

at 14°C in G.S.M. were transferred to a suitable medium preequilibrated at 30°C. Initially, studies were carried out on the effect of such a transfer on the viability of the cells using a plating technique, and later the biochemical basis or bases of the loss of viability at 30°C were studied by comparing changes in the chemistry of the cell on exposure to 30°C. The test temperature of 14°C was attained in the Psychrotherm incubator mentioned, 30°C in a New Brunswick Metabolyte Water Bath Shaker, equipped also with a rotary shaker. The pattern of the test varied slightly with the material to be studied. Where high cell densities were required, as when the leakage of small amounts of intracellular leakage was under investigation, cells grown at 14°C were harvested, washed three times at 0°C in S.M., pH 7.5, and then resuspended in S.M. at 30°C. This procedure was adopted for most of the chemical tests on the cell, the effect of added glutamic acid being recorded when studied. For experiments where a dilute suspension of cells in a large volume was required, 10 ml. of a suitable suspension of cells at 14°C were pipetted directly into 90 ml. of S.M. or G.S.M. (pH 7.5) at 30°C. Where only a small volume was required at 30°C, 10 ml. of cells grown at 14°C were pipetted directly into a sterile, thin-walled flask in the waterbath at 30°C. In this case it was observed that the temperature of the culture rose from 14°C to over 25°C in approximately 10 seconds and reached 30°C in 25 to 30 seconds. In all studies, the

test cells were harvested in the early-to midlogarithmic phase of growth and always a control culture was maintained at 14°C. The relevance of the findings in each test to the viable state of the culture was made clear by sampling for viability at intervals throughout the test.

The criterion of viability in this study was the ability of the cell under study to reproduce and form colonies. The viable count was used throughout, with one exception where optical density was employed, as alternative methods such as measurement of changes in turbidity, or viable staining, proved to be too tardy in relation to the expression of the temperature-sensitive lesion and, in the case of the latter, cumbersome and inaccurate. One drawback of the method employed is that the test organism is a micrococcus, irregularly grouped, but largely in the diplococcus form, and a colony count does not reflect the exact numbers of cells present but rather the number of colony-forming units. This is not significant should the distribution of groupings remain unchanged throughout the test but if an elevated temperature promotes cellular clumping then erroneous results will be obtained. To verify this point the effect of medium-power sonic oscillation (Branson Sonifier Model S-75, Branson Instruments Inc., Darbury, Conn.) on cells which had been exposed to 30°C for some time was measured. Short bursts of oscillation at this power will not affect the viability of control cells at 14°C, but should break up any artificially formed clusters.

Turbidity Changes on Transfer of Cells to 30°C and Back to 14°C

Cultures of ATCC 15174 grown at 14°C were transferred to 30°C at various points along the growth curve and incubated at the higher temperature for periods of 1, 2 and 3 hours before being returned to the 14°C incubator. Changes in $O.D._{600}$ during and following the transfer period were observed.

Isolation and Study of Mesophilic Mutants

The wild strain, ATCC 15174, was grown at 14°C in G.S.M. for 24 hours and the cells were harvested on membrane filters (Gelman Metricel: 0.45 microns pore size) and washed with cold S.M. The filters were laid on the surface of TSA plates and exposed for periods of 1 to 10 seconds to ultraviolet irradiation. The covers were placed on the plates and the cultures placed in a 30°C incubator for 48 hours. Colonies appearing on the filter surfaces were picked for sub-culture into TSB at 30°C. Ten mutants, capable of growth at 30°C, were isolated and compared with the wild-type by colonial morphology, staining, sugar fermentation and the other standard biochemical classification tests. A mutant was sought as similar to the psychrophile as possible in most of its characters including growth on G.S.M. but, unlike the wild strain, growing at temperatures above 25°C. From these investigations, one mutant (15174-RB) was retained for further examination.

II. STUDIES ON THE EFFECT OF SPECIFIC SUPPLEMENTATION ON VIABILITY.

The Effect of the Presence of Nutrients on Viability at 30°C

To determine whether any supplementation of S.M. had any specific effect in modifying the extreme temperature-sensitivity of ATCC 15174, the known synthetic supplements prepared previously were re-employed.

Alternatively, cells incubated at 30°C in S.M. were plated out on agar containing S.M. with the addition of several of the nutrients used previously. For comparison, control plates of G.S.A. were also utilised and the results compared.

III. STUDIES ON MEMBRANE INTEGRITY

Measurement of Intracellular Material Leaked From the Cell

Cells were suspended in S.M., pH 7.5, at either 30°C or 14°C, at a final concentration of 0.5 mg. dry weight/ml. At regular intervals samples were withdrawn, tested for viability and the cell-free supernatant examined for the presence of any leaked cellular products.

Measurement of Ultraviolet-Absorbing Compounds

The supernatants were scanned in the U.V. region, from 225 millimicrons to 325 millimicrons, in a Bausch and Lomb 505 recording spectrophotometer.

Protein Estimation

Chemical analysis of samples began with an estimation of protein content by the Folin method of Lowry *et al*. (1951). A suitable 0.5 ml. dilution of sample was mixed with 5 ml. of a mixture of 1 part 0.5% copper sulphate in 1% sodium tartrate to 50 parts 2% Na_2CO_3 in 0.1 N NaOH and then incubated for 30 minutes at 40°C to allow the Biuret reaction of protein with Cu^{++} in alkali to take place. Five ml. of 1 N Folin reagent were added to allow the second stage of the reaction - reduction of the phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan present in the heated protein - to proceed and the intensity of the blue colour was measured at 500 millimicrons in a Spectronic 20. A standard curve was prepared using bovine serum albumin (Calbiochem).

Ribonucleic Acid Estimation

Following protein estimation the samples were deproteinised by heating for 30 minutes at 90°C in an equal volume of 10% trichloracetic acid (TCA) and filtering through TCA-washed Whatman Number One filter papers to obtain a clear protein-free supernatant. The RNA contents of the samples were estimated by measuring the orcinol-reacting compounds present (Schneider, 1957). A suitable 1.5 ml. dilution of nucleic acid extract was mixed with 1.5 ml. of 1% orcinol dissolved in 100 ml. conc. HCl containing 0.5 g. $FeCl_3$. This mixture was heated for 20 minutes in a boiling waterbath and the intensity of the green colour read at 660 millimicrons in a Spectronic 20. A Standard curve was prepared using yeast RNA (Calbiochem) and, since DNA also reacts with the reagent, a calibration curve was prepared for this material using salmon sperm DNA (Calbiochem).

Deoxyribonucleic Acid Estimation

The DNA present was estimated by measuring the diphenylamine-reacting compounds present in the deproteinized extract. Two ml. of a 1% solution of diphenylamine in reagent glacial acetic acid and reagent concentrated sulphuric acid were mixed with 1 ml. of a suitable dilution of the material and, after heating for 10 minutes in boiling water, the intensity of blue colour read at 600 millimicrons in a Spectronic 20 (Schneider, 1957). A standard curve was prepared using salmon sperm DNA (Calbiochem).

Amino Acid Estimation

The ninhydrin-reacting compounds present in the deproteinised extract were estimated as described by Spies (1957).

One-tenth of a ml. of a suitable dilution of the sample was added to 1 ml. ninhydrin reagent (2% ninhydrin in 50 parts methyl cellosolve plus 50 parts 0.2 M citrate buffer, pH 5.0, containing 0.16% $SnCl_2.2H_2O$) and the mixture heated for 20 minutes in a boiling waterbath. Five ml. of diluent (equal volumes water and n-propanol), were added to each tube, mixed and the intensity of the purple colour at 570 millimicrons read after 15 minutes in a Spectronic 20. A standard curve was prepared using samples of L-leucine.

Alpha-keto Acid Estimation

The hydrazones formed by the reaction of alpha-keto acids and 2,4-dinitrophenyl hydrazine were estimated as described by Friedemann (1957). Three ml. of sample were brought to 25°C in a waterbath and 1 ml. of reagent (0.5% 2,4-dinitrophenyl hydrazine in 2N HCl) added. The mixture was incubated for 25 minutes then 8 ml. ethyl acetate were added. The contents of the tube were aerated for 2 minutes with a rapid stream of air then allowed to separate out into two phases of which one, the aqueous phase, was discarded. Six ml. of a 10% Na_2CO_3 solution in water were added, the mixture aerated for 2 minutes and then 5 ml. of the lower carbonate extract transferred to a colorimeter

tube in a 25°C waterbath. On the addition of 5 ml. 1.5 N NaOH an orange-red colour, whose intensity was read after 5 minutes at 435 millimicrons in a Spectronic 20, was produced. The keto acid concentrations were calculated from a standard curve prepared using alpha-ketoglutarate.

Electron Microscope Studies

Preparations of ATCC 15174 incubated in S.M. at 30°C for varying periods of time were examined under the electron microscope for any obvious signs of cellular damage due to temperature effects. Control cultures at 14°C were also examined and the findings were related to the viability of the cultures at any specified time.

Initially shadow casts were prepared according to the method of Bradley (1965) using gold-palladium alloy as the casting material. Samples from a culture containing approximately 0.5 mg. dry weight of cells/ml. were dried on a carbon film and placed on a glass slide in a shadow caster. The prepared specimens were examined and photographed in a Phillips Model EM-100 electron microscope.

A negative staining technique with potassium phosphotungstate (Thornley and Horne, 1962) was employed in the hope that more details of the fine structure might be made visible. Support grids were covered with a thin film of nitrocellulose and stabilized by an additional layer of evaporated carbon. A solution of 2% (w/v) phosphotungstic acid was adjusted with KOH to pH 7.2 and samples from a similar culture to the one described above were suspended in this solution to give a concentration of about 0.2 mg.

dry weight/ml. cells. A drop of this suspension was placed on the prepared supports, and the surplus fluid removed with filter paper, leaving a thin film covering each grid. On drying, the films were ready for examination.

Fluorimetry

Aqueous solutions of 8-anilino-1-napthalene sulphonic acid (ANS) do not fluoresce when excited by ultraviolet light; in the presence of protein the dye combines with negatively-charged groups and the conjugate fluoresces strongly when excited by light of wavelength 346 millimicrons (Weber and Laurence, 1954). When washed cells were suspended in dilute solutions of this dye no fluorescence was observed, indicating there were no groups on the surface of the cell with which the dye could combine. Treatment with a membrane-binding antibiotic, however, resulted in increasing fluorescence, presumably the alteration in cellular permeability allowing the dye to penetrate the cell and combine with cell protein (Newton, 1954). The rate of membrane damage at 30°C can be determined, and compared with loss of cellular viability, by measuring the intensity of fluorescence under these conditions in the presence of ANS.

The sodium salt of 8-anilino-1-naphthalene sulphonic acid was obtained from Eastman Kodak Co. (Rochester, N.Y.) and purified by precipitation with ammonium sulphate, the ammonium salt coming down as an oil which crystallised after

several days.

Intensities of fluorescence were measured in a Turner III Fluorometer (G.K. Turner and Assoc., Palo Alto, Calif.), fitted with a 110-811 filter, giving an exciting wavelength of approximately 350 millimicrons, and a 110-817 filter giving a recording wavelength greater than 470 millimicrons.

The procedure employed was a modification of that adopted by Newton; fluorescence was described as a percentage of the range between an untreated, washed suspension of ATCC 15174 and one killed by exposure to 60°C for 5 minutes. Cells were suspended in S.M. at a final concentration of 0.02 mg. dry weight/ml. At hourly intervals up to 8 hours, 3 ml. samples were withdrawn and to these were added ANS, in sodium chloride at pH 7.0, to a final concentration of 50 millimicromoles/ml. This concentration of the dye had been shown previously to be non-toxic to the cells. The final mixture was maintained at 10°C in a waterbath and 1 minute allowed for fluorescence development.

IV. STUDIES ON INTERMEDIARY METABOLISM

Isolation, Characterisation and Quantitation of The Constituents of the Amino Acid Pool

Cells grown at 14°C and cells grown at 30°C were harvested, washed and suspended in distilled water to a concentration of 10 mg. dry weight/ml. and the amino acid pools extracted by placing the suspension in a boiling waterbath for 15 minutes. The cell-free supernatant was retained for examination after the total amino acid content had been estimated by the method of Spies (1957).

The constituents of the amino acid pools from these samples were separated and measured by two-dimensional paper chromatography (Rockland and Underwood, 1957). Five-inch squares of Schleicher and Schuell No. 507 Blue Ribbon filter papers were spotted 5/8 inch from the sides of the lower left corner with 10 lambda of cell extract. Reference chromatograms were prepared using standard amino acid solutions (0.06 M). The filter papers were mounted on suspending rods, so that the machine direction was parallel to the solvent flow, and suspended in American Medical Museum jars containing approximately 75 ml. of Solvent I. Solvent I has the following composition: 10 ml. formic acid (specific gravity 1.2) and 295 ml. of deionized water were mixed with 695 ml. of *tert*.-butyl alcohol at 24°C. The solvent was allowed to migrate to within 1/4 inch of the upper edge of the paper, approximately 9 hours irrigation.

After drying overnight at room temperature, the chromatograms were observed under ultraviolet light for uniformity of the solvent boundary. The distance travelled by Solvent I was measured and recorded in pencil along a vertical edge of the filter paper. After trimming the boundary, each chromatogram was remounted with the vertical, pencilled edge adjacent and parallel to the suspending rod, and inserted in a museum jar containing approximately 75 ml. of Solvent II. Solvent II has the following composition: 775 g. molten phenol (60°C) and 215 g. deionised water mixed as a stock solution stored in the dark. Just prior to use 94 ml. of this solution were mixed with 1 ml. concentrated ammonium hydroxide (specific gravity 0.9) at 24°C. Less than 4 hours were required for irrigation with Solvent II.

At least three replicate chromatograms of each test substance were prepared, care being taken to place the unknown replicates in separate jars together with replicate chromatograms of other standards. All separations were carried out at approximately 20°C.

The positions of the amino acids on the paper chromatograms were detected with a ninhydrin reagent (0.5% ninhydrin in a mixture of 10 parts glacial acetic acid, 20 parts of anhydrous pyridine, 30 parts of methyl cellosolve and 40 parts of tert.-butyl alcohol by volume) using dip staining.

The Rf values were calculated from the spot centre and solvent boundary distances measured to the nearest millimetre. The spots were then cut out and eluted in 3 ml.

reagent grade n-propanol and deionised water (1:1), and the colour density of the solution compared with a standard curve prepared from known concentrations of the same amino acids similarly separated and eluted.

The results are expressed as amount of amino acid/mg. dry weight of cells.

Isolation, Characterisation and Quantitation of The Constituents of the Alpha-keto Acid Pool

The cell extract was examined for the presence of alpha-keto acids by the method of Berry and Campbell (1964). A volume of 0.05 ml. extract was applied in portions with gentle heating to a 6 mm. filter paper disc impaled on a pin. The dry disc was then inserted in a slit in a dry 40 x 410 mm. Schleicher and Schuell filter paper strip which was moistened in 0.125 M veronal buffer, pH 8.6, and placed in the cassette of an LKB paper electrophoresis apparatus No. 3276B (LKB-Produkter AC, Stockholm, Sweden). Electrophoresis of the sample was carried out for 3.5 hours at 25°C using a current of 10 milliamps, following which the strips were dried and sprayed with 0.5% 2,4-dinitrophenylhydrazine in 2 N HCl. After 3 minutes development, the strips were examined for the appearance of yellow spots representing the DNPH derivatives of the acids formed directly *in situ* on the paper. These zones were cut out and eluted in 5 ml. of 40% KOH, the supernatants being collected by filtration and their optical densities at 380 millimicrons

determined using a Beckman Model DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The concentrations of the unknowns were estimated from a curve prepared using known concentrations of alpha-ketoglutaric acid in 10-lambda volumes handled in the same manner. A reference mixture containing alpha-ketoglutaric acid and pyruvic acid was run with each unknown. The results are expressed as amount of keto acid/mg. dry weight of cells.

Respirometry

The respiratory activities of ATCC 15174 at 14°C and 30°C, in the presence and absence of exogenous glutamic acid, were determined as described by Umbreit, et al. (1964) with the constant volume Warburg respirometer (Precision Scientific Co., Chicago, Ill.) fitted with cooling coils through which was circulated water from a low temperature waterbath when required.

A suspension containing between 5 mg. and 10 mg. dry weight/ml. of cells was prepared and 0.5 ml. was added to a clean, dry Warburg flask. The total volume was adjusted to 3.0 ml. by adding 2.0 ml. S.M., pH 7.5, to the main compartment, and 0.5 ml. S.M. or glutamic acid solution (3%, w/v; pH 7.5) to the side arm, depending upon whether the respiration of endogenous or exogenous reserves was being measured. The centre well of each flask contained 0.2 ml. 20% (w/v) KOH. After the Warburg flask had been attached to the manometer, it was equilibrated in the waterbath for 10 minutes and then the manometer tap was closed

and the substrate tipped over from the side arm. The uptake of oxygen by the organisms was followed over a period of at least two hours.

The respiratory activities are expressed as either oxygen uptake (micromoles O_2 consumed/mg. dry weight of cells) or as QO_2 values (micromoles O_2 consumed/mg. dry weight of cells/hour). These activities were determined either for the respiration of endogenous reserves, or of exogenous substrates after the values for endogenous respiration had been subtracted.

Estimation of Adenosine-5'-Triphosphate Content

The effect of temperature on the synthesis of adenosine-5'-triphosphate (ATP) by cells of ATCC 15174 growing either at 14°C or 30°C was determined by measurement of the bioluminescence produced by the reaction between ATP and the enzyme luciferase in the presence of O_2 and Mg^{++}(Strehler, 1963). Cells were suspended to a concentration of 0.3 mg./ml. in either S.M. or G.S.M. at 14°C and 30°C over an eight hour period during which time samples were withdrawn, 1 ml. for a viable count of the cells, and 8 ml. to be centrifuged down for ATP extraction. This was achieved by boiling the cells for 15 minutes in deionized water and then retaining the supernatant at 20°C for examination. The Michaelis constant for ATP is 5×10^{-5} M at pH 7.4. At ATP concentrations which are a little below the Michaelis half saturation value, the rate of the over-all reaction is proportional to the ATP

concentration, and a curve showing the relationship between light intensity and ATP concentration, using dilutions of 1.6×10^{-4} M solution of ATP-$Na_2H_3.3H_2O$ (Calbiochem.), was prepared. As the reaction reaches its maximum rate almost immediately and then decreases essentially as a first order process with time as ATP is consumed, preparatory studies on the time course of luminescence in the system were carried out and a measurement time of 20 seconds after mixing adopted. The luciferase extract was prepared by grinding 50 mg. of vacuum-dried firefly lanterns (Sigma) with 5 ml. ice cold 0.1 M sodium arsenate buffer, pH 7.4, for 10 minutes. The mixture was filtered into a test-tube standing in an ice cold bath of 50 mg. $MgSO_4.\ 7H_2O$ was added to the filtrate. The extract was stored at 4^oC and used within 24 hours. A 0.2 ml. quantity of the luciferase preparation was diluted to 0.6 ml. with distilled water and the reaction initiated by the injection of 0.2 ml. cell extract or ATP standard, by syringe. Following rapid mixing, the light intensity was read in a Beckman DU spectrophotometer at the specified time and the reading compared with that on the standard curve.

Preparation of Cell-free Extracts

Extracts of organisms for use in the measurement of enzyme activities were prepared by sonic oscillation in a Branson Sonifier, model S-75, fitted with a Rosett cooling cell. Cells were harvested and washed twice with 0.05 M phosphate buffer, pH 7.5, at 0°C and suspended to a final concentration of 20 mg./ml. dry weight of cells in ice-cold water in the cooling cells along with 1g:10 ml. of glass beads (Minnesota Mining & Manufacturing Co : Superbrite Pavement Marking Beads, 100 microns diameter) which had been washed with concentrated nitric acid prior to use. Sonication was carried out at full power for two four-minute periods during which time the cooling cell was held in a methanol - dry ice cooling bath at approximately -10°C. Under these conditions the temperature of the suspension within the cell remained around 0°C - 2°C. Cell-free extracts were obtained by centrifuging the suspension of disrupted organisms at 25,000 g. for 10 minutes at 0°C. Extracts were either used immediately or stored at -20° until required. The protein contents of the extracts were determined by the method of Lowry et al. (1951) with bovine serum plasma albumin (Calbiochem.) as a standard.

Assay of Enzymes of Intermediary Metabolism

The enzyme nomenclature is that recommended in the Report of the Commission on Enzymes of the International Union of Biochemistry, 1961, although only the suggested

trivial names are used for the dehydrogenases studied since the experimental results do not permit precise identification of the enzymes concerned. The activities for all the enzymes studied were calculated from initial velocities determined over a period during which plots of the amount of substrate changed against time were linear. All activities were calculated as specific activities (micromole substrate consumed/mg. protein/hour).

The activities of citrate synthase, aconitate hydratase, isocitrate dehydrogenase, fumarate hydratase, L-malate dehydrogenase, isocitratase, malate synthetase, glutamic dehydrogenase, glutamic-oxaloacetate-transaminase, and glutamic-pyruvic-transaminase were measured spectrophotometrically in a Beckman DB-G Grating Spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.) fitted with a constant temperature cuvette housing. The reactions were carried out in 1 cm. quartz cuvettes and unless otherwise stated, all reaction constituents except the cell extract were equilibrated in the cuvettes at the test temperature before starting the reaction. The temperature of the reaction mixture in the cuvettes was measured by using a YSI Telethermometer Model 42SL (Yellow Springs Instrument Co., Yellow Springs, Ohio), fitted with a hypodermic thermistor. The biochemicals utilized were obtained from either Sigma Chem. Corp. or Calbiochem. Citrate synthase (citrate oxaloacetate-lyase (Co A-acetylating); E.C.4.1.3.7.) activity in cell extracts was determined by the method of Dixon and Kornberg (1959). This involved measuring the rate of

decrease of optical density at 232 millimicrons consequent upon breakage of the thio-ester bond of acetyl coenzyme A in the presence of oxaloacetate (Stadtman, 1957). Each cuvette contained, in 2.1 ml, tris buffer (90 micromoles; pH 7.1) and acetyl coenzyme A (0.125 micromoles; pH 7.1). One-tenth ml. of cell extract containing approximately 100 micrograms protein was added to the test cell and the change in extinction at 232 millimicrons recorded for several minutes to detect the possible presence of acetyl coenzyme A deacylase. Cis-oxalacetate (5 micromoles) was then added; the initial rate of the rapid decrease in optical density is proportional to the citrate synthase present. The specific activities were calculated from the change in extinction at 232 millimicrons for the thio-ester bond of acetyl coenzyme A which is 4.5×10^3 (Stadtman, 1957).

Aconitate hydratase (citrate (isocitrate) hydrolyase; E.C.4.2.1.3.) was assayed by a method based on that of Racker (1950) which depends upon measuring the increase in extinction at 240 millimicrons attendant upon the conversion of citrate to cis-aconitate. Each cuvette was charged with sodium citrate (87 micromoles; pH 7.4) and sodium phosphate buffer (145 micromoles; pH 7.4) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of a portion of cell extract containing approximately 100 micrograms protein; 0.1 ml. water was added to the control cuvette. The increase in extinction at 240 millimicrons, caused by the formation of cis-aconitate, was followed at 30-second

intervals for a period of 5 minutes. Specific activities were calculated using the value for the extinction in 3.0 ml. water of 1 micromole cis-aconitate at 240 micromoles quoted by Williams and Rainbow (1964).

Isocitrate dehydrogenase ($NADP^+$-linked) was assayed by following at 340 millimicrons the increase in extinction on reduction of $NADP^+$ (Ochoa, 1948: Kornberg and Pricer, 1951). Each cuvette contained sodium DL-isocitrate (0.5 micromole; pH 7.0), potassium phosphate buffer (100 micromoles; pH 7.0), $NADP^+$ (0.5 micromole), $MgCl_2$ (10 micromoles) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of cell extract containing about 100 micrograms protein, and the increase in extinction at 340 millimicrons was followed at 30-second intervals over a period of 3 minutes, with a blank reaction mixture lacking cell extract. The extinction of a second blank reaction mixture containing all of the constituents except DL-isocitrate was measured at the beginning and at the end of the period of observation. Specific activities were calculated from the change in extinction using the molar extinction coefficient for $NADPH.H^+$ quoted by Horecker and Kornberg (1948).

Fumarate hydratase (L-malate hydrolyase; E.C.4.2.1.2.) activity was assayed using a method based on that of Racker (1950) which depends on measuring the decrease in the extinction at 300 millimicrons attendant on the conversion of fumarate to L-malate. Each cuvette contained sodium fumarate (49 micromoles; pH 7.3) and sodium phosphate buffer

(95 micromoles; pH 7.3) in 2.9 ml. water. The reaction was started by adding 0.1 ml. cell extract containing about 400 micrograms protein; 0.1 ml. water was added to the control reaction mixture. The decrease in extinction at 300 millimicrons, caused by the decrease in the concentration of fumarate, was followed at 30-second intervals over a period of 5 minutes. Specific activities were calculated using a value for the extinction of 1 micromole fumarate in 3.0 ml. at 300 millimicrons of 0.015.

L-malic dehydrogenase (NAD^+-linked) activity was assayed using oxaloacetate as substrate and following the decrease in extinction caused by the oxidation of $NADH.H^+$ (Ochoa, 1955a). Each cuvette contained sodium phosphate buffer (150 micromoles; pH 7.4), potassium oxaloacetate (0.76 micromole; pH 7.4) and $NADH.H^+$ (0.15 micromole; pH 7.4) in 2.9 ml. water. The reaction was started by adding 0.1 ml. of cell extract containing about 100 micrograms protein and the decrease in extinction at 340 millimicrons was followed at 30-second intervals over a period of 3 minutes. Specific activities were calculated using the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948).

Iso-citratase (L_S-isocitrate glyoxylate-lyase; E.C.4.1.3.1.) was assayed by measuring the rate of increase of optical density at 324 millimicrons consequent upon the formation of glyoxylic acid phenylhydrazone from glyoxylate produced by the action of this enzyme from iso-citrate, and

phenylhydrazone (Dixon and Kornberg, 1959). Each cuvette contained sodium phosphate buffer (150 micromoles; pH 6.9), $MgCl_2$ (15 micromoles; pH 6.9), phenylhydrazine HCl (10 micromoles; pH 6.9), cysteine HCl (6 micromoles; pH 6.9), in 2.9 ml. water. The reaction proceeded on addition of 5 micromoles potassium iso-citrate and after a lag of approximately 1 minute, the change in extinction at 324 millimicrons was linear for about 4 minutes the rate being proportional to the enzyme concentration. Specific activities were calculated using the molar extinction coefficient for glyoxylic acid phenylhydrazine of 1.7×10^4 at 324 millimicrons.

Malate synthetase (L-malate glyoxylate lyase (Co A-acetylating); E.C.4.1.3.2.) activity was assayed by measuring the rate of decrease of optical density at 232 millimicrons consequent upon breakage of the thio-ester bond of acetyl CoA (Stadtman, 1957) in the presence of glyoxylate (Dixon and Kornberg, 1959). The reaction system contained in 2.9 ml., tris buffer (90 micromoles; pH 7.1), $MgCl_2$ (10 micromoles; pH 7.1), acetyl coenzyme A (0.125 micromole; pH 7.1), and 0.1 ml. of cell extract containing approximately 100 micrograms protein. The change in optical density at 232 millimicrons was recorded for several minutes to detect the possible presence of an acetyl coenzyme A deacylase and then 5 micromoles sodium glyoxylate were added. The quantity of malate formed in the reaction mixture corresponds to the amount of acetyl CoA utilised and the specific activities were calculated using a molar

extinction coefficient at 232 millimicrons of 4.5×10^3 for the cleavage of the thio-ester bond of acetyl coenzyme A (Stadtman, 1957).

Glutamic dehydrogenase (NAD^+- or $NADP^+$-linked) activities were assayed in two ways by measuring either the decrease in optical density at 340 millimicrons as the reduced coenzyme is oxidised, in the case of the synthetic direction (Vender et al, 1965), or by the increase in extinction attendant upon the formation of reduced coenzyme as glutamate is oxidised. The synthetic reaction system contained tris buffer (90 micromoles; pH 7.5), $(NH_4)_2SO_4$ (40 micromoles; pH 7.5), alpha-ketoglutaric acid (6.7 micromoles; pH 7.5), $NADPH.H^+$ or $NADH.H^+$ (0.15 micromoles; pH 7.5) in 2.9 ml. The reaction was initiated by the addition of 0.1 ml. cell extract containing about 100 micrograms protein and the change in optical density at 340 millimicrons was followed at 30-second intervals over a period of 4 minutes. Specific activities were calculated using the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948). The oxidation of glutamate in the presence of oxidised coenzyme was measured according to Strecker (1955). Each cuvette was charged with phosphate buffer (150 micromoles; pH 7.6), NAD^+ or $NADP^+$ (0.1 micromole; pH 7.6) potassium glutamate (100 micromoles; pH 7.6) in 2.9 ml. water and 0.1 ml. cell extract containing approximately 100 micrograms protein. The increase in absorption at 340 millimicrons was recorded at 30-second intervals.

Glutamic-oxaloacetic-transaminase (L-aspartate: 2-oxoglutarate aminotransferase; E.C.2.6.1.1.) activity was assayed by the use of a coupled reaction whereby the oxaloacetate formed by the transaminase action is catalysed by malic dehydrogenase (L-malate: NAD^+: oxidoreductase; E.C.1.1.1.37) in the presence of $NADH.H^+$ to form malate plus oxidised coenzyme, (Bergmeyer and Bernt, 1963). The oxidation of $NADH.H^+$, which is proportional to the amount of oxaloacetate formed, is measured by the decrease in optical density at 340 millimicrons. Each cuvette contained sodium phosphate buffer (150 micromoles; pH 7.5), L-aspartate (125 micromoles; pH 7.5), $NADH.H^+$ (0.12 micromoles; pH 7.5), malic dehydrogenase, (diluted in ammonium sulphate to approximately 2000 units), and cell extract (approximately 100 micrograms protein) to a final concentration of 2.9 ml. The reaction mixture was incubated for about 10 minutes at the assay temperature until there was no further change in optical density, and then 0.1 ml. alpha-ketoglutarate (20 micromoles; pH 7.5) was added and the decrease in extinction recorded at 340 millimicrons at 1-minute intervals for about 5 minutes. Specific activities were calculated from the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948).

Glutamic-pyruvic-transaminase (L-alanine; 2 oxoglutarate aminotransferase; E.C.2.6.1.2.) activity was assayed by employing lactic dehydrogenase (D-lactate: NAD^+ oxidoreductase) as an indicator enzyme to catalyse the reduction

of the pyruvate formed on transamination to lactic acid with the concomitant oxidation of $NADH.H^+$. The rate of oxidation of this coenzyme is proportional to the increase of pyruvate with time and is measured by the decrease of optical density at 340 millimicrons (Bergmeyer and Bernt, 1963). Each cuvette was charged with sodium phosphate buffer (150 micromoles; pH 7.4), L-alanine (55 micromoles; pH 7.4), $NADH.H^+$ (0.12 micromole; pH 7.4), lactic dehydrogenase (approximately 800 units) and cell extract (approximately 100 micrograms protein) to a final volume of 2.9 ml. The mixture was equilibrated for about 10 minutes at the assay temperature until the optical density had stabilized and then alpha-ketoglutarate (20 micromoles; pH 7.4) was added and the decrease in optical density at 340 millimicrons followed at 1-minute intervals for 5 minutes. Specific activities were calculated from the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948).

The activities of pyruvate, alpha-ketoglutarate and succinate dehydrogenases, along with glutamic decarboxylase, were assayed manometrically by the conventional constant volume respirometer technique (Umbreit et al, 1964). Pyruvate dehydrogenase activity was assayed by measuring carbon dioxide evolution with sodium pyruvate as substrate and potassium ferricyanide as electron acceptor (Jaganathan and Schweet, 1952). Each flask was charged with sodium pyruvate (100 micromoles), sodium bicarbonate (50 micromoles),

$MgCl_2$ (20 micromoles) and thiamine pyrophosphate (200 micrograms; adjusted to pH 6.0 just before use). These constituents were added as a solution which had been flushed with carbon dioxide gas for 2 to 3 minutes. A portion of cell extract containing approximately 5 mg. protein and water to a volume of 3 ml. were also added to the flask, and water to a volume of 3 ml. were also added to the flask, the side arm of which contained potassium ferricyanide (100 micromoles). The flasks were attached to the manometers, and flushed with carbon dioxide gas for 10 minutes, with a glass manifold to ensure even gassing. The manometer units were then quickly transferred to the waterbath and equilibrated for 10 minutes. After the potassium ferricyanide had been tipped in from the side arm, the evolution of carbon dioxide was followed during a period of 10 minutes. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Alpha-ketoglutarate dehydrogenase activity in cell extracts was assayed by measuring carbon dioxide evolution in a system which contained alpha-ketoglutarate as substrate and potassium ferricyanide as electron acceptor (Sanadi et al, 1952). The main compartment of each Warburg flask was charged with alpha-ketoglutarate (20 micromoles). These constituents were added as a solution that had been flushed for 2 to 3 minutes with carbon dioxide gas. The solution in the Warburg flasks were supplemented with bovine

plasma albumin (30 mg.), a portion of cell extract containing approximately 5 mg. protein, and water to a volume of 3 ml. The side arm of the flask contained potassium ferricyanide (50 micromoles). The flasks were attached to the manometers, flushed with carbon dioxide gas for 10 minutes, quickly transferred to the Warburg bath and equilibrated for 10 minutes. After the potassium ferricyanide had been added from the side arm, the evolution of carbon dioxide was recorded during 10 minutes. Control flasks contained all the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Succinic dehydrogenase activity in cell extracts was assayed by measuring the amount of oxygen uptake with succinate as a substrate in the presence of phenazine methosulphate as an electron carrier (Bernath and Singer, 1962). Each Warburg flask was charged with sodium phosphate buffer (150 micromoles; pH 7.6), KCN (30 micromoles; pH 7.6), cell extract containing approximately 3 mg. of protein and water to 3 ml. Sodium succinate (60 micromoles; pH 7.6) and phenazine methosulphate (0.2, 0.1, 0.07, 0.05 or 0.04 ml. of a 1%, w/v, solution) were added to the side arm. KCN was added last, the flasks immediately attached to the manometers and the stopcocks closed. The excess pressure was released momentarily after the units had been placed in the Warburg bath. After equilibrating for 10 minutes the contents of the side arms were tipped into the flasks and

the uptake of oxygen was recorded during 20 minutes. Control flasks contained all of the constituents of the reaction mixture except cell extract. The reciprocal of the QO_2 value (calculated over a period of 2 to 12 minutes) was then plotted against the reciprocal of the concentration of phenazine methosulphate and the oxygen uptake extrapolated to infinite phenazine methosulphate concentration. This value for the oxygen uptake was used to estimate the specific activities of succinic dehydrogenase in the extracts (Bernath and Singer, 1962).

Glutamic decarboxylase (L-glutamate 1-carboxy-lyase; E.C.4.1.1.15) activity was assayed by measuring carbon dioxide evolution in a system which contained L-glutamic acid as the substrate and pyridoxal phosphate as the co-factor (Vender et al, 1965). The main compartment of each Warburg flask was charged with pyridoxal phosphate (0.30 mg.) and acetate buffer (180 micromoles; pH 4.8) in the form of a solution that had been flushed with a carbon dioxide gas for 2 to 3 minutes. The solution in the flask was supplemented with cell extract, containing approximately 2 mg. protein, bringing the total volume up to 3 ml. The side arm of the flask contained L-glutamic acid (45 micromoles). The flasks were attached to the manometers, flushed with carbon dioxide for 10 minutes, quickly transferred to the Warburg bath and equilibrated for 10 minutes. After the L-glutamate had been tipped in from the side arm, the evolution of carbon dioxide was recorded during 10 minutes.

Control flasks contained all the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Aspartase activity was assayed by measuring the ammonia gas produced by the action of the enzyme on aspartic acid (Vender et al, 1965). Thin-walled glass tubes were charged with tris buffer (100 micromoles; pH 7.5), L-aspartic acid (5 micromoles; pH 7.5), $MnCl_2$ (5 micrograms) and cell extract, containing about 100 micrograms protein, in a total volume of 2 ml. The reaction was allowed to proceed for 15 minutes and then halted by the addition of 0.2 ml. 50% trichloracetic acid (TCA). The precipitate was spun down and the supernatant assayed for ammonium nitrogen produced using Nessler's reagent (Johnson, 1941). The nitrogen in the sample was calculated by use of a standard curve prepared using NH_4Cl standards containing between 1 and 50 micrograms nitrogen. The control reaction mixtures contained no cell extract. Specific activities were calculated from the rate of transformation of substrate per mg. cell extract protein.

Detection and Measurement of Incorporation of Radioactive Precursor

The relative uptake and incorporation of a labelled precursor which had been fed, at $14^{o}C$, to normal cells and cells exposed for a short period of time to moderate temperature, was detected and measured using the fractionation techniques of Canvin and Beever (1961). Washed cells of a

normal culture of ATCC 15174 grown at 14°C, and those of a culture which had been exposed to 30°C for 90 minutes leading to an approximate 50% loss of viability, were suspended to a concentration of 5 mg. dry weight/ml. in 0.05 M phosphate buffer, pH 6.5, and distributed in 2 ml. amounts to separate Warburg flasks, in duplicate. A 1.0 ml. quantity 20% KOH was added to the centre well and the flasks attached to manometers in the waterbath at 14°C and the system allowed to equilibrate. At time zero the experiment was started by adding 0.2 ml. $^{14}CH_3COONa$ (1 micromole; 2 microcurie, New England Nuclear Corporation), the stoppers tightened and the reaction allowed to proceed for 20 minutes. At the end of this period the flasks were opened in a fume cupboard and the reaction halted by the addition of 2 ml. boiling 95% ethanol. The labelled carbon dioxide evolved during the course of the reaction and absorbed by the alkali of the centre well was removed by a syringe and collected, along with the distilled water washings of the well. The flask was then laid on a hot plate and the cell suspension plus ethanol allowed to boil for about 30 seconds.

The contents of the syringe were then supplemented with an excess of barium ions (1 ml. 10% $BaCl_2$) to precipitate the $^{14}CO_2$ in the syringe. This precipitate was collected by passing the whole sample through a membrane filter unit (Gelman Metricel filter; 1.2 micron pore size) which retained the insoluble labelled carbonate. The filters were air dried, glued to a planchet and the activity read

in a thin-end-window gas flow counter (Nuclear-Chicago). The counts were corrected for self-absorption and background radioactivity using controls.

The cellular fraction was washed out from the Warburg flask with ethanol and the insoluble material separated from the soluble material by centrifugation at 25,000 g. The supernatant, supernatant 1, was retained. The cell debris was resuspended in 50% ethanol, recentrifuged and supernatant 2 separated from the pellet. Finally, the pellet was washed in water, spun down as before and the third supernatant fraction was pooled with numbers 1 and 2. The pellet was termed the insoluble residual fraction and the pooled supernatants the alcohol-water soluble fractions. These were fractionated and examined as follows.

The insoluble residual fraction was hydrolyzed in 6 N HCl containing 0.1% stannouschloride for 20 minutes at 112°C and 15 lbs. pressure. Following hydrolysis the insoluble residue was collected on a glass-fibre filter paper disc and dried in an oven at 100°C for 6 hours. The dried material and an aliquot of soluble hydrolysate were assayed for activity as before. These constitute the insoluble residual fraction.

The pooled supernatants were concentrated by flash evaporation (Buchler Instruments Ltd., Fort Lee, N.J.) below 40°C and aliquots of the material were dried on planchets and assayed for radioactivity. Following treatment with 1% CH_3COOH, the planchets were counted once more, any difference

in counts representing either unused $^{14}CH_3COONa$, or volatile organic acids or perhaps carbonates in the soluble fractions.

The alcohol-water soluble fraction was separated further on the basis of charge using ion exchange chromatography. The first separation took place on 6 cm. x 1 cm. columns of Dowex AG 50W-X8 resin (hydrogen form; 200-400 mesh) (Calbiochem.). The resin was packed and washed with deionised water until the pH of the effluent reached 7.0. The supernatant fraction was applied to the column which was then washed with 40 ml. deionised water and the effluent, containing sugars and organic acids, was collected. Elution of the column with 40 ml. 4 N NH_4OH released the basic fraction, amino acids. Each fraction was concentrated, the activity of aliquots measured and then rechromatographed to give further separation. This time columns were generated from Dowex AG 1 x 10 (Chloride form; 200-400 mesh) (Calbiochem.). Again a water slurry of resin was used to pack 6 cm. by 1 cm. columns which were washed with deionised water until the effluent was neutral. Acetate and formate forms of the resin were generated from the chloride form by passing 1 M solutions of sodium acetate or sodium formate through the column of resin until the effluent was chloride-free (as indicated by the silver nitrate test). The chloride-free resins were then washed with 40 ml. 0.1 M acetic acid (or 0.1 M formic acid in the case of the formate resins) before being neutralised with deionised water. After

this final washing, the soluble fractions were separated as follows: an aqueous solution of the mixture of sugars plus organic acids was introduced to the top of the formate column and the neutral sugars were washed through with 40 ml. deionised water and the organic acids eluted later with 40 ml. 4 N formic acid; an aqueous solution of the amino acid mixture, all NH_4OH having been evaporated off, was introduced to the top of the acetate column and the neutral and basic amino acids were washed through with 40 ml. deionised water while the acidic amino acid fraction was eluted later with 40 ml. 4 N acetic acid. In this latter separation the amides, glutamine and aspargine are collected with the neutral and basic amino acid fraction. These final fractions were again evaporated to smaller volumes and aliquots collected and dried on planchets for the measurement of activity.

The complete fractionation procedure is summarised in Figure 1 and Figure 2.

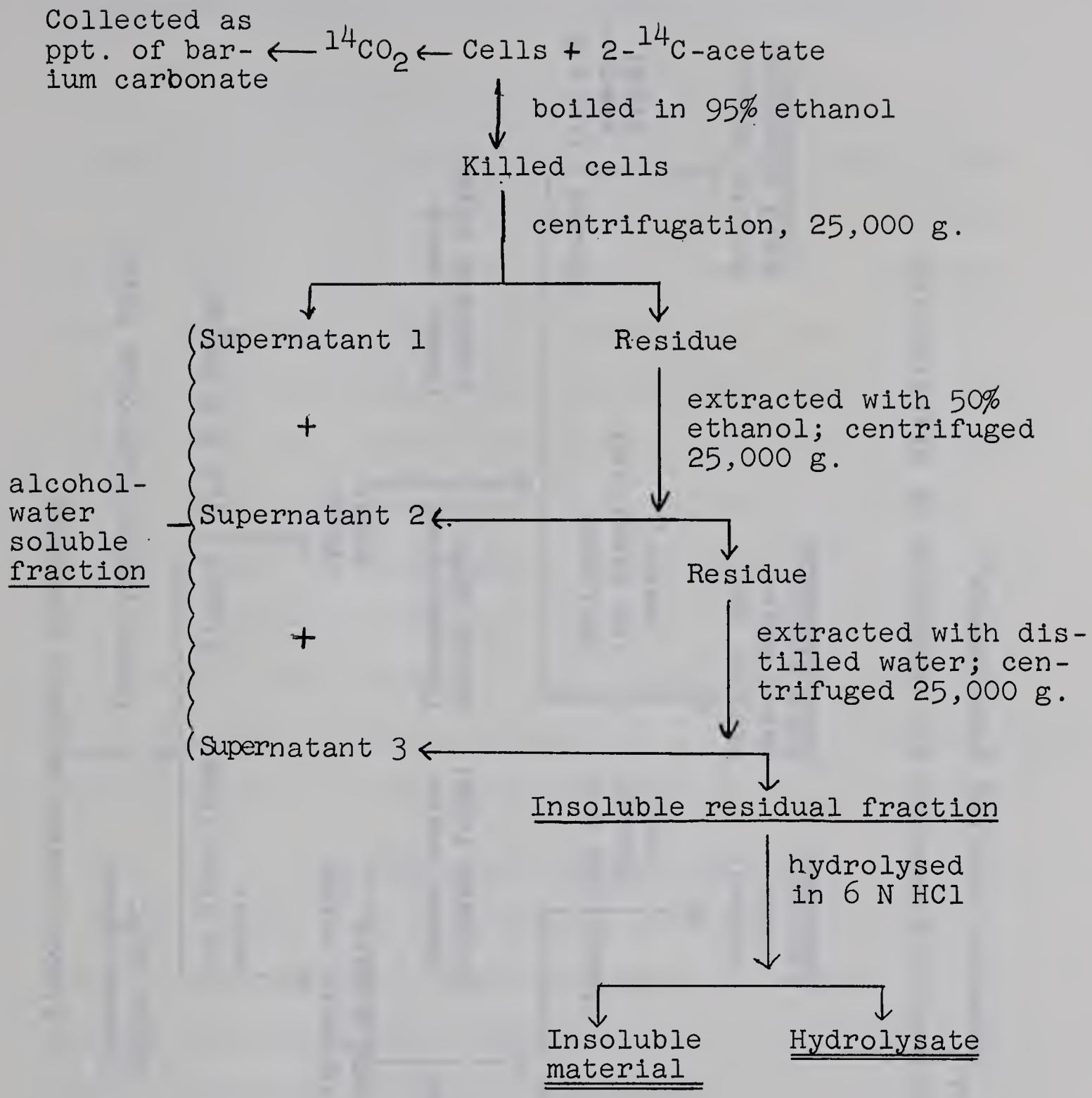
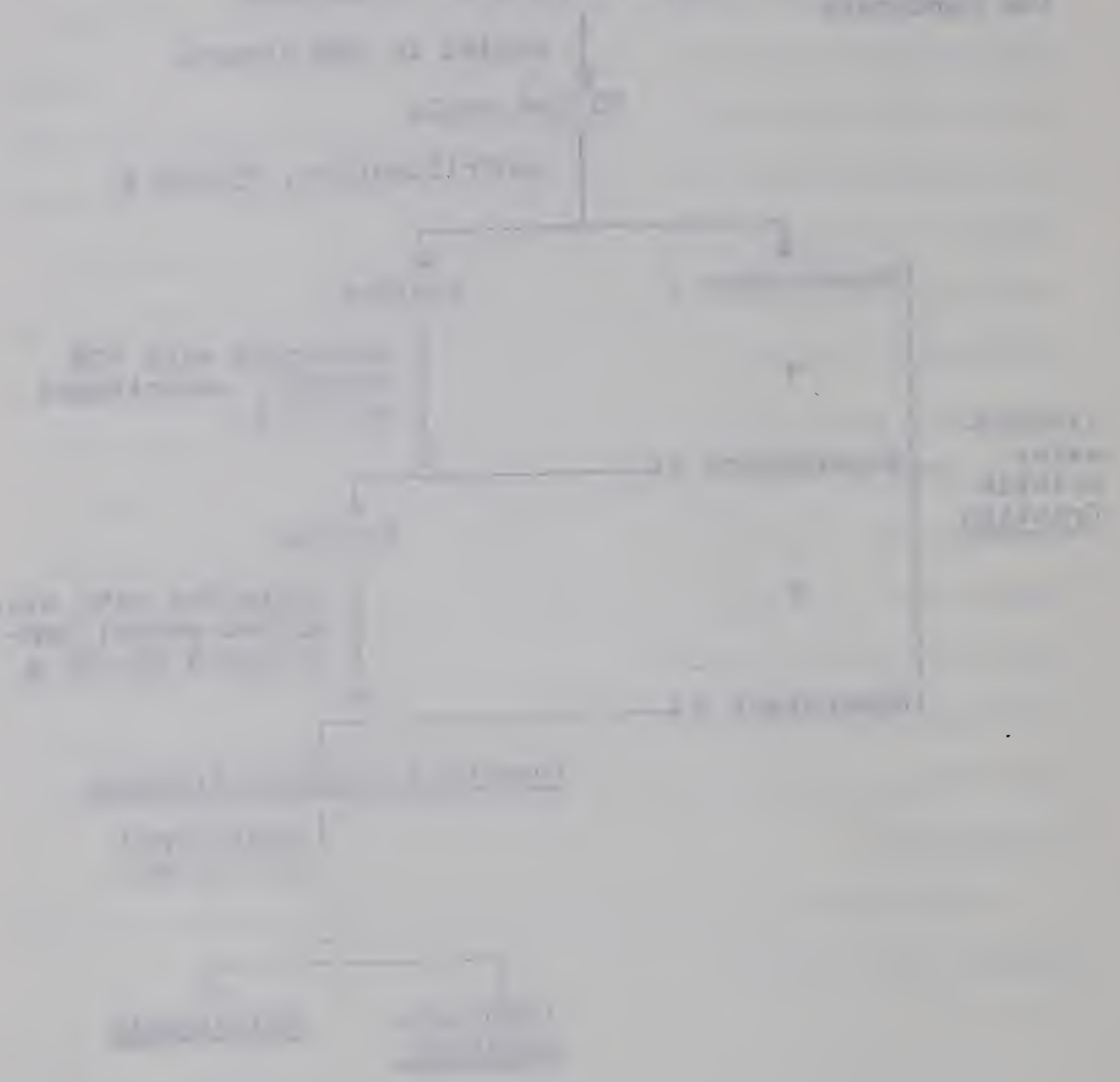


Fig. 1. Initial fractionation procedure for detection of distribution of label in 2-^{14}C-acetate-feeding experiments.



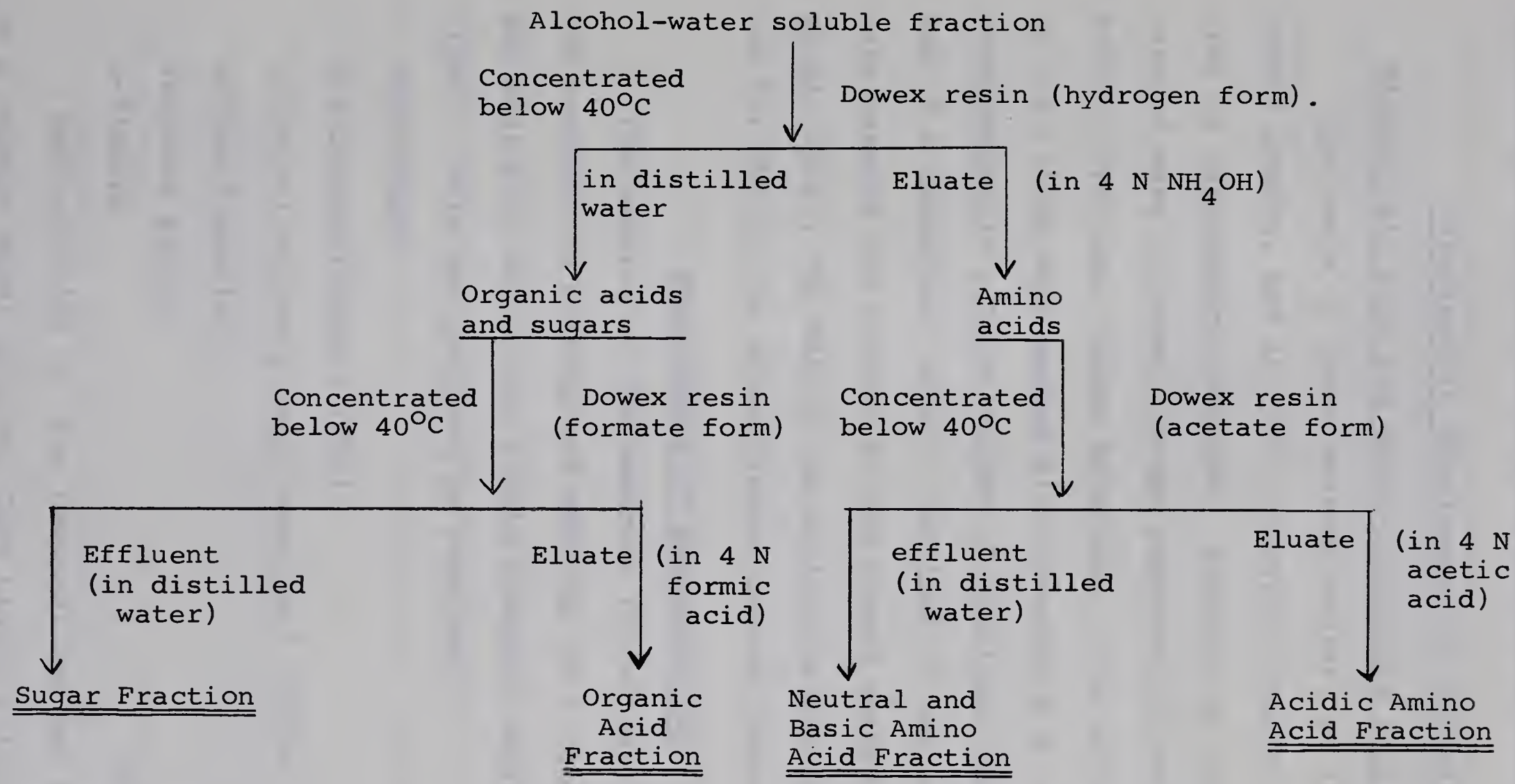
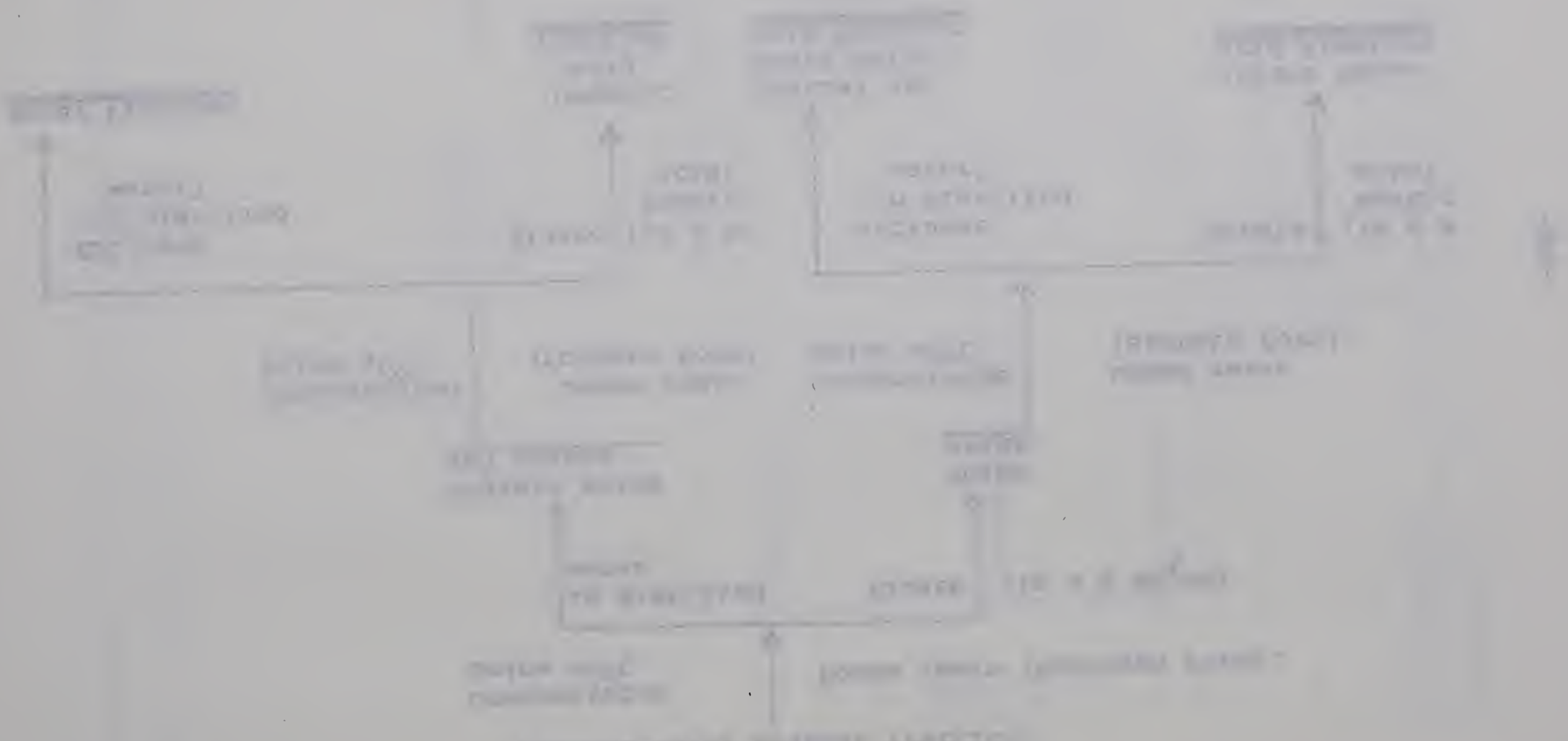


Figure 2: Secondary Fractionation Procedure for Detection of Distribution of Label in 2-^{14}C-acetate Feeding Experiments.



V. STUDIES ON MACROMOLECULAR SYNTHESIS AND BREAKDOWN

Colorimetric Measurement of Protein, Ribonucleic Acid and Deoxyribonucleic Acid Synthesis

Synthesis of these macromolecules, at 14°C during normal growth, and at 30°C following transfer, was measured by colorimetric methods. Proteins of culture to be assayed were filtered through membrane filters (.80 micron pore size; Gelman Metricel). The cells collected on the filter were washed with ice-cold S.M. and then resuspended in a known volume of distilled water with the aid of a mechanical mixer. Portions of the suspension were assayed for protein by the phenol method (Lowry, et al. 1951), for RNA by the orcinol method (Schneider, 1945), and for DNA by the diphenylamine method (Schneider, 1957).

Measurement of Radioactivity

The samples on the membrane filter were prepared for counting by placing the material in a counting vial and adding 10 ml. of the liquid phosphor devised by Bray (1960). This was prepared by combining:

naphthalene	60.0 g.
2,5-diphenyloxazole (PPO)	4.0 g.
1,4-bis-(2-5-phenyloxalyl-benzene) (POPOP)	0.2 g.
methanol (absolute)	100 ml.
ethylene glycol	20 ml.
p-dioxane	to 1 litre

Radioactivity of the preparations was determined in a Packard Model 3003 Tri-Carb Liquid Scintillation

Spectrometer fitted with an Automatic Control, Model 574. Quenching was determined by counting an internal standard of ^{14}C-cholesterol and the radioactivity in the samples was expressed as counts per minute in the 1 ml. sample withdrawn.

Incorporation of ^{14}C-leucine Into Protein

Cells were suspended in G.S.M., pH 7.5, at either 14°C or 30°C to a final concentration of 0.02 mg. dry weight/ml. The growth medium was supplemented with 80 micrograms/ml. leucine containing a total of 0.2 microcurie of L-leucine-1-^{14}C (Calbiochem). During incubation samples were withdrawn and the reaction terminated immediately by the addition of an equal volume of ice-cold 10% trichloracetic acid (TCA) and the mixture held in an ice-bath overnight. The precipitate formed was collected on a membrane filter (Gelman Metricel; 0.45 micron pore size), washed twice with cold 5% TCA and twice with cold 1% TCA. The filter was air dried and the activity present in the precipitate estimated and recorded as a measurement of the incorporation of ^{14}C-leucine into proteins. (Roodyn and Mandel, 1960).

Incorporation of ^{14}C-uracil Into Nucleic Acids

The method was similar to that described for the incorporation of ^{14}C-leucine with the exception that G.S.M. was supplemented with 40 micrograms/ml. uracil containing a total of 0.2 microcurie uracil-2-^{14}C (Calbiochem.) in place of the labelled amino acid.

Measurement of Transport of Leucine and Uracil Into Cells

Cells were suspended in G.S.M., pH 7.5, at either 14°C or 30°C to a final concentration of 0.02 mg. dry weight/ml. The growth medium was supplemented with either 80 micrograms/ml. leucine containing a total of 0.2 microcurie ^{14}C-leucine, or 40 micrograms/ml. uracil containing a total of 0.2 microcurie ^{14}C-uracil. One ml. samples were collected at intervals on a 0.45 micron pore size membrane filter and washed quickly with 5 volumes of ice-cold SM. The filter was dried in a 40°C incubator and the radioactivity present in the cells estimated and recorded as a measurement of the transport of leucine or uracil into the cells.

Measurement of Nucleic Acid 'Turnover'

Two methods were employed:

a) The method developed by Tarver (1954) based on the rate of disappearance of radioactivity from a prelabelled material to which a non-radioactive precursor has been added. This method will be referred to as the 'kinetic method'. It has been shown that such a process can be considered to be a first-order reaction and, therefore, the following kinetic equation can be used for the evaluation of turnover:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.639}{k.}$$

Where k is the specific reaction rate and $t_{1/2}$ is the half-life period of the decay process. If the time required to remove the amount of the material equivalent to the size of the cellular pool is t_t, then according to Tarver:

$$t_t = t_{1/2} \times \frac{1}{\ln 2} = 1/k.$$

As the value for k can be obtained from the experimental data, t_t can be calculated.

The reaction mixture contained 0.2 mg. dry weight of cells in 10 ml. G.S.M., pH 7.5, at 14°C plus 0.2 microcurie ^{14}C-uracil (0.2 micromole). This mixture was incubated for 1 hour and then 40 micrograms/ml. non-radioactive analogue were added. One ml. samples were collected at intervals and the radioactivity determined in the cold TCA-insoluble material.

b) The method for measuring the turnover of messenger RNA (mRNA) by Bubela and Holdsworth (1966) is referred to as the 'time-interval method'. It is based on the following assumptions: 1) Nucleic acids are involved in protein synthesis. 2) mRNA has the highest turnover of all nucleic acids. 3) uracil can be incorporated into RNA. 4) mRNA synthesis is inhibited by actinomycin D. Experimental evidence for all of these assumptions exists (Levinthal *et al*, 1963). A further assumption is made that if the synthesis of the RNA stops, then mRNA synthesis will cease and any further incorporation of amino acids into proteins must be due to the "surviving" mRNA. If the time interval necessary for

actinomycin D to inhibit mRNA synthesis is known and similarly if the time interval, before amino acid incorporation into proteins is affected due to lack of mRNA, is measured, the life span of mRNA can be estimated. The following values have to be known: 1) The time lag before actinomycin D affects RNA synthesis and such a time interval is designated as t_u. 2) The time lag before actinomycin D affects amino acid incorporation. Such a time interval is designated t_{aa}.

The lag period for the action of actinomycin D in mRNA synthesis was estimated by pre-incubating for 15 minutes 0.2 mg. dry weight of cells in 10 ml. G.S.M. at 14^o in the presence of 0.2 microcurie ^{14}C-uracil. One-tenth ml. of actinomycin D (Mercke, Sharpe and Dohme), giving a final concentration of 10 micrograms/ml. was added and 1 ml. samples were removed at intervals, treated with cold 10% TCA and the precipitate collected on membrane filters. The activity of the samples was measured and the time at which the time-activity graph changed its slope distinctly was taken as the measure of the lag period. This time was designated t_u.

The lag period for the action of actinomycin D on protein synthesis was estimated by adding 10 micrograms/ml. (final concentration) to the previously described reaction mixture containing 0.2 microcurie of uniformly labelled leucine instead of uracil. Samples were collected at various times after the addition of actinomycin, treated

with cold TCA, the precipitate collected and the radioactivity measured. The time lag period obtained in the same way as that for RNA synthesis was designated t_{aa}.

The value for the life span was obtained from the formula $t_t = t_{aa} - t_u$.

Effect of Addition of Chloramphenicol On Incorporation of ^{14}C-uracil

Chloramphenicol blocks protein synthesis quickly and completely but permits continued synthesis of RNA. (Gale and Folkes, 1953; Wisseman et al, 1954). The effect of halting protein synthesis on the incorporation of ^{14}C-uracil was studied at 14°C and 30°C. Cells of ATCC 15174 was suspended in G.S.M., pH 7.5, to a concentration of 0.02 mg. dry weight/ml. and supplemented with 0.2 microcurie ^{14}C-uracil in a final concentration of 40 micrograms/ml. uracil. At time zero, or at a specified interval later, enough chloramphenicol (Parke, Davis and Company, Ltd.) was added in 0.1 ml. to give a final concentration of 60 micrograms/ml. One ml. samples were withdrawn at fixed times through the test and the uracil incorporated into synthesized nucleic acid determined as before by estimating the radioactivity in the cold TCA-insoluble protein.

Assay of Amino Acid - Transfer - Ribonucleic Acid Synthetases

Amino acid-transfer ribonucleic acid (tRNA) synthetases catalyze a reaction between ATP and various specific

amino acids leading to the activation of the amino acid and the elimination of phosphate or pyrophosphate from ATP. These enzymes were assayed by allowing the activated amino acid to react with hydroxylamine (NH_2OH) under relatively mild conditions yielding the amino acyl hydroxamate which can be measured colorimetrically with ferric chloride (Stulberg and Novelli, 1962). The reaction mixture contained, in 1 ml., tris buffer (200 micromoles, pH 7.8), ATP (10 micromoles), amino acids (10 micromoles), salt-free NH_2OH (1000 micromoles) and either cell extract containing approximately 100 micrograms of protein or water. Incubation proceeded for one hour and then the reaction was halted by the addition of 2.3 ml. of colour assay reagent (0.37 M $FeCl_3$, 0.31 M TCA, and 0.65 M HCl). The precipitated protein was removed by centrifugation and the colour density at 540 millimicrons read in a Spectronic 20 and compared with a standard curve obtained by using a synthetic amino acid hydroxamate in the above procedure in the absence of enzyme. To lessen the problem of endogenous amino acid saturation of the enzymes, the cell extracts were dialyzed overnight against 0.006 M tris buffer (pH 7.5) plus 0.006 M mercaptoethanol at 1°C. Specific activity was calculated as micromoles hydroxamate formed/mg. protein/hour.

A synthetic amino acid hydroxamate (glycinehydroxamic acid) was prepared according to Safir and Williams (1952). To an ice-cold solution of 0.1 M glycine ethyl ester and 0.1 M of hydroxylamine chloride in 17 ml. water, 0.33 M of

NaOH was slowly added with constant stirring to give a final volume of 43.4 ml. This solution was acidified with 8.5 ml.12N HCl and on cooling in ice, the product crystallized as a white solid which was filtered and washed with a little cold water.

Salt-free hydroxylamine was prepared by the method of Beinert et al. (1953). Fifty-six grams of NH_2OH HCl were dissolved in 200 ml. absolute methanol and slowly mixed with 60 g. KOH dissolved in another 200 ml. of methanol, to give a final pH of 7.5 to 8.0. The solution was chilled to 0°C, and the KCl removed by filtration. Two hundred grams of anhydrous Na_2SO_4 were then added, and the salt again removed by filtration after 2 to 3 hours at 0°C. The solution was concentrated to a thin syrup in a rotating flash evaporator and the concentration determined by titration with HCl. The final pH was adjusted by the addition of HCl so that a 1:10 dilution read pH 7.8 on a pH meter. The solution was then diluted to 10.0 M by the addition of water and stored at -10°C.

Fractionation of Ribonucleate Released Into Medium at 30°C.

Washed cells of ATCC 15174 were divided and suspended, half at 14°C and half at 30°C, in 0.05 M phosphate buffer, pH 7.5, to a final concentration of 1.8 mg. dry weight/ml. A four-hour incubation period was selected as it had been shown earlier that within this time significant amounts of 260 millimicron-absorbing and orcinol-reacting material

appeared in the supernatant of cells at 30°, with little or no contamination by other cellular components. The cells were then spun down and the supernatants frozen and retained for examination along with a lysate of normal cells which had been prepared by sonic oscillation. The RNA materials were analysed in two ways, chemically and physically.

1) Chemical analysis: suitable aliquots of supernatant and of the cell lysate were mixed with an equal volume of cold 10% TCA and the precipitate removed by centrifugation. The supernatant was increased to a fixed volume with 5% TCA and retained (cold supernatant). The precipitate was washed once with cold 5% TCA, suspended in a fixed volume of 5% TCA and heated at 90°C for 30 minutes to bring the polymeric RNA into solution. After centrifugation the supernatant was retained (hot supernatant). These two fractions were adjudged to contain degraded RNA and polymeric RNA respectively (Schneider, 1945) and orcinol determinations were carried out on each to determine the distribution and levels of orcinol-reacting materials in the extracts. The ratios of the types of RNA found were calculated.

2) Physical analysis: Sephadex column chromatography was carried out with a Pharmacia column (Pharmacia, Uppsala, Sweden), gravity packed with Sephadex G-25 (Pharmacia) fine bead gel to bed dimensions of 39 cm. x 2.54 cm., using deionised water as the solvent and eluent. Pressure was maintained with a Sigmamotor and a flow rate of 82.5 ml. per hour

was obtained. Dextran 2000 (Pharmacia) was used to determine the void volume of the column. Five millilitre sample volumes were washed into the column surface and fractions were collected every 150 seconds (3.0 ml.) by a Beckman Model 132 Fraction Collector (Beckman Instruments Inc., Fullerton, Calif.) and the optical density at 260 millimicrons read for each lute. The elution patterns were studied for both supernatants, cell lysate and yeast RNA.

Characterization of Ribonucleate Released at 30°C

From gel filtration a fraction of 260 millimicron-absorbing and orcinol-reacting material, constituting around 95% of the total orcinol-reacting compounds released from the cell, was detected in the 30° supernatant only. These findings suggested selective leakage and the fraction was examined further by the methods of Singh and Lane (1964). The fraction was desalted by charcoal adsorption and elution, recovery being around 90%. Paper chromatographic resolution of the sample was achieved by first impregnating Whatman No. 1 paper by dipping into a solution made by diluting 1 volume of saturated ammonium sulphate solution with 9 volumes of water. After air-drying, the papers were spotted in the usual manner and chromatograms were developed with a solvent composed of 80 volumes of 95% ethanol and 20 volumes of water. Preconditioning was achieved with 4 ml. of 95% ethanol. The chromatograms were run 24 hours at room temperature, the samples being separated along with

markers consisting of mixtures of known nucleotides and nucleosides. The separated spots were examined first under U.V. light and recorded by U.V. photography.

Assay of Ribonuclease Activity

The hydrolysis of RNA by ribonuclease (RNase) was measured by the formation of free acid groups which were not precipitable in TCA (McDonald, 1955). Reaction mixtures were prepared containing, in 1.9 ml., sodium phosphate buffer (150 micromoles; pH 7.5) and RNA (4 mg.) Cell extract (0.1 ml.) containing about 100 micrograms protein was added to the mixtures which were incubated at appropriate temperatures for 12 hours. The reaction was terminated by the addition of 2 ml. ice-cold 10% TCA and the acid-soluble fraction was collected by centrifugation. The increase in orcinol-reacting compounds in the acid-soluble fraction was measured over controls containing no RNA. The specific activity was expressed as milligrams of acid-soluble material released per mg. protein per hour.

RESULTS

Nutrient Requirements of ATCC 15174

Of the several factors thought to be involved in determining the maximum temperature for growth, enzyme denaturation has been considered one of the most likely. A search for thermolability among the catalytic activities of the cell can be aided greatly by an initial knowledge of the basic nutrient requirements of the organism. This study commenced with an attempt to determine chemically defined minimal media capable of supporting the growth of ATCC 15174. This was done by replacing complex mixtures of nutrients with known compounds, individually or in mixtures and noting their ability to substitute for the original.

The nitrogen requirement of ATCC 15174 was found to be satisfied by the inclusion of L-glutamic acid in the medium as the sole nitrogen source. This could be replaced, but much less successfully, by L-proline suggesting perhaps that the rate of growth in this case is limited by the efficiency with which L-proline is degraded to L-glutamate. Aspartic acid and the other amino acids did not support growth. The purity of the commercial preparation of L-glutamate available was checked by the two-dimensional chromatography method of Rockland and Underwood (1954). No traces of other amino acids were found.

No purine, pyrimidine or vitamin requirements were found for this organism; although the presence of certain

of these did stimulate growth slightly, their absence was not fatal to the cell.

Similarly, no specific carbohydrate requirement was found, the growth rate of the cells seemingly unchanged no matter which carbohydrate was present or whether any carbohydrate was present at all. The possibility that ATCC 15174 cannot utilise D-glucose was investigated a) by including some of this sugar in the medium and studying the growth response in relation to a control culture incubated without glucose; b) by measuring the amount of D-glucose present in the cell-free supernatant at various stages of growth. The results, recorded in Table 1, indicate that the presence of glucose in the medium has no effect on the growth of ATCC 15174 which does not appear to utilise it. From this it is obvious that ATCC 15174 is capable of growth on L-glutamate in a buffered medium, plus the presence of a few salts. Even citrate added as a chelating agent to the salts base is not utilised by the cell, as a replacement of the phosphate buffering system with tris buffer which requires no chelating agent, and hence no citrate, made no difference to the growth response. No attempt was made to define more rigorously the mineral requirements of the cell.

These results suggest that ATCC 15174 has no hexose-permease system or an incomplete glycolytic pathway, at least in the catabolic direction. Halpern and Umbarger (1961), in studying ammonia metabolism in Escherichia coli,

Table 1: Effect of Added D-Glucose on the Growth of ATCC 15174

Time of Incubation at 14°C (Hours)	$O.D._{600}$; G.S.M. + D-Glucose	$O.D._{600}$; G.S.M.*	Residual supernatant glucose in supplemented medium
0	0.003	0.003	990 micrograms/ml.
6	0.003	0.003	980 " "
12	0.004	0.004	1000 " "
24	0.017	0.018	990 " "
36	0.12	0.125	985 " "
48	0.82	0.84	990 " "

*Glutamate salts medium.

noted that wild-type strains of this organism could not grow on glutamate as sole source of carbon. These workers selected mutants capable of utilising glutamate as sole source of carbon, and concluded that the mutation had enhanced the permeability of the mutants to glutamate; they also noted that the mutants had partially or completely lost glutamic decarboxylase activity. Vender et al. (1965) describe the isolation of similar mutants and present evidence suggesting that the mutation leading to the loss of glutamic decarboxylase activity endows the bacteria with the ability to utilise glutamate as sole source of carbon. Gilvarg and Davis (1956) also isolated a mutant of E. coli which, by virtue of its inability to form citrate, required glutamate for growth. In the present study glutamate is a requirement for the wild-type organism and this is not due, as will be shown later, to a lack of either condensing enzyme or of glutamic decarboxylase activity.

The findings support the evidence of McLean et al. (1951) who, in the first taxonomic description of this organism, noted that it is not capable of growth in sodium citrate and no fermentation of 9 different sugars occurred. Their report, however, that inorganic nitrogen is incapable of supplying the nitrogen requirement of M. cryophilus is contradicted by some of the results in a study carried out on possible alternatives to glutamic acid in the medium. Table 2 shows, once more, that proline but not aspartic acid can replace glutamic acid alone; alpha-ketoglutarate

Table 2: Effect of Replacement of Glutamate in Minimal Medium on Growth of ATCC 15174 at 14°C

Carbon Source	Nitrogen Source	$O.D._{600}$ After 48 Hours
Glutamate	Glutamate	0.380
Proline	Proline	0.125
Aspartate	Aspartate	0
alpha-ketoglutarate	Aspartate	0.150
alpha-ketoglutarate	NH_4+	0
alpha-ketoglutarate	NO_3^-	0
Pyruvate	Aspartate	0.050
Pyruvate	NH_4+	0
Pyruvate	NO_3^-	0
Malate	Aspartate	0.005
Malate	NH_4+	0
Malate	NO_3^-	0
Acetate	Aspartate	0
Acetate	NH_4+	0.020
Acetate	NO_3^-	0.005

or pyruvate, in the presence of aspartate, though not inorganic nitrogen, will support growth; and, surprisingly, acetate plus NH_4^+ or NO_3^- can sustain growth but not acetate plus aspartate. Growth on acetate suggests a glyoxylate cycle in the coccus induced by the presence of this 2C-acid and no 4C-acids. Under these conditions, but not otherwise, mechanisms for the incorporation of inorganic nitrogen into cellular compounds might become available. All of these substitutions yielded much slower growth rates for ATCC 15174 than did glutamic acid alone.

Micrococcus cryophilus is a strict aerobe and growth in liquid media is greatly enhanced by shaking; therefore the most probable route for the dissimilation of glutamic acid to yield energy is probably the deamination of the amino acid - by transamination reactions as suggested by the results in Table 2 - to alpha-ketoglutarate which is then oxidised by the enzymes of the Krebs' Cycle. As the reactions of the Embden-Meyerhoff pathway do not serve as catabolic route in this organism but in a purely biosynthetic capacity, the Krebs' Cycle plays a vital, 'amphibolic' (Davis, 1961) role in metabolism fulfilling equally functions in energy release and supply of biosynthetic precursors.

This minimal medium, glutamate-salts-medium (GSM) supported growth of the psychrophile over the entire temperature range, -4°C to 25°C, as did complex medium.

Growth Characteristics of ATCC 15174 and 15174-RB On Glutamate-Salts-Medium

Figure 3 shows the growth curve for ATCC 15174 at 14°C in G.S.M. with aeration. Four subcultures, from the log phase, had taken place before the measurements began and this reduced the lag phase to around eight hours. The mean generation time in the exponential phase of growth is five hours and the phase itself lasts for approximately forty hours. A calibration curve relating $O.D._{600}$ to dry weight of cells is presented in Figure 4.

The pH of the growth medium, buffered initially at 7.2, rises in conjunction with the logarithmic phase and becomes quite alkaline, reaching 8.3 by the stationary phase of growth (Figure 5).

The mesophilic mutant, 15174-RB, was selected from the other mutants on the similarity of its taxonomic properties to those cited by McLean et al (1951) for the parent strain, and for its ability to grow in G.S.M. Only the upper growth limit of the cell appeared to be changed as cultures grew below 0°C and up to a maximum of 34°C and, in one instance, on continued subculturing at 5°C in G.S.M. it was observed to revert to the obligately psychrophilic state. The growth curves for this mutant at 14°C and 30°C in G.S.M. are illustrated in Figure 6. The rate of growth at 14°C initially exceeds that at 30°C during a short, exponential phase of approximately 20 hours when the mean generation time is 5.5 hours, close to that of the parent.

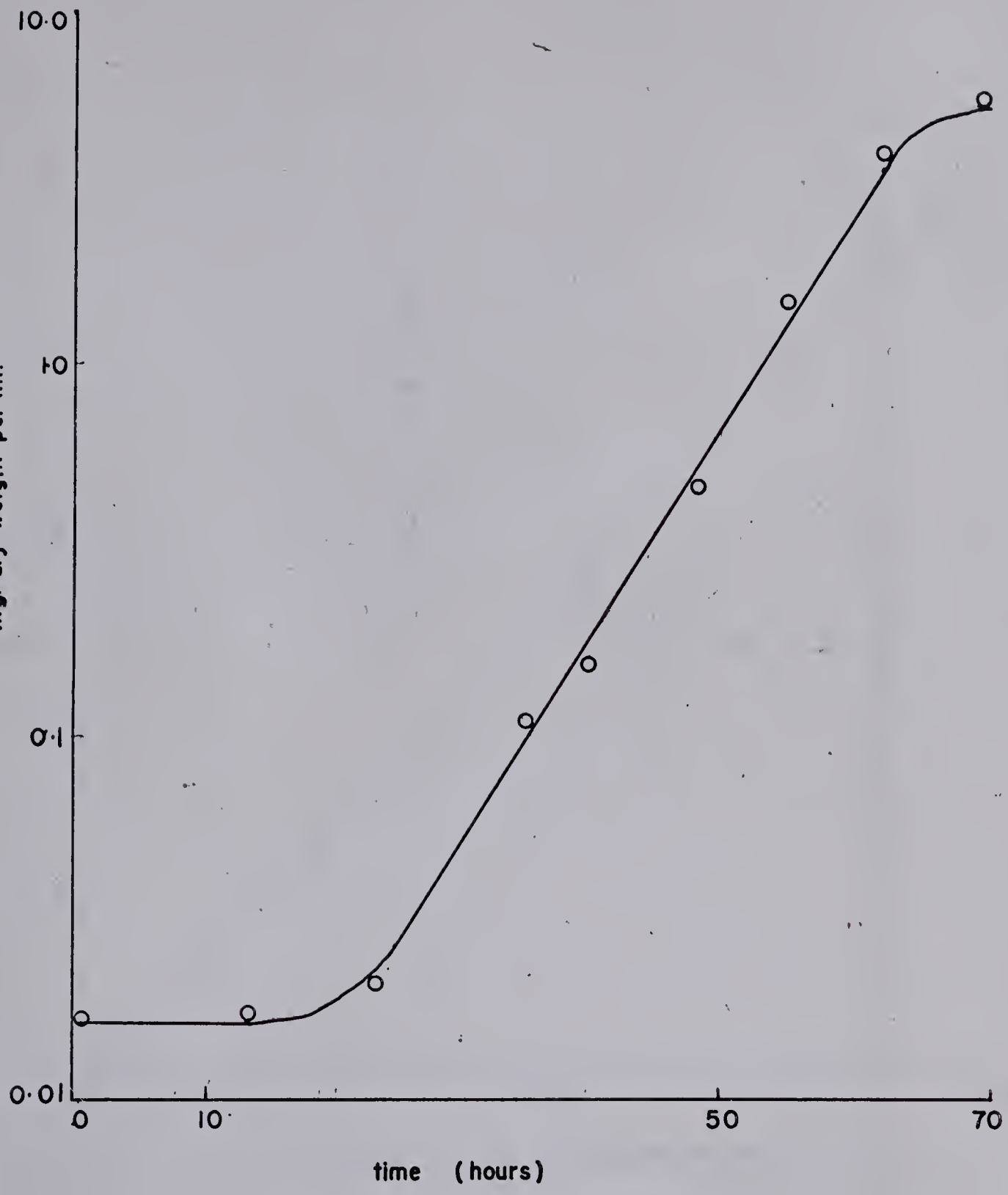


FIG. 3. GROWTH CURVE FOR ATCC 15174 IN GLUTAMATE SALTS MEDIUM AT 14°C.

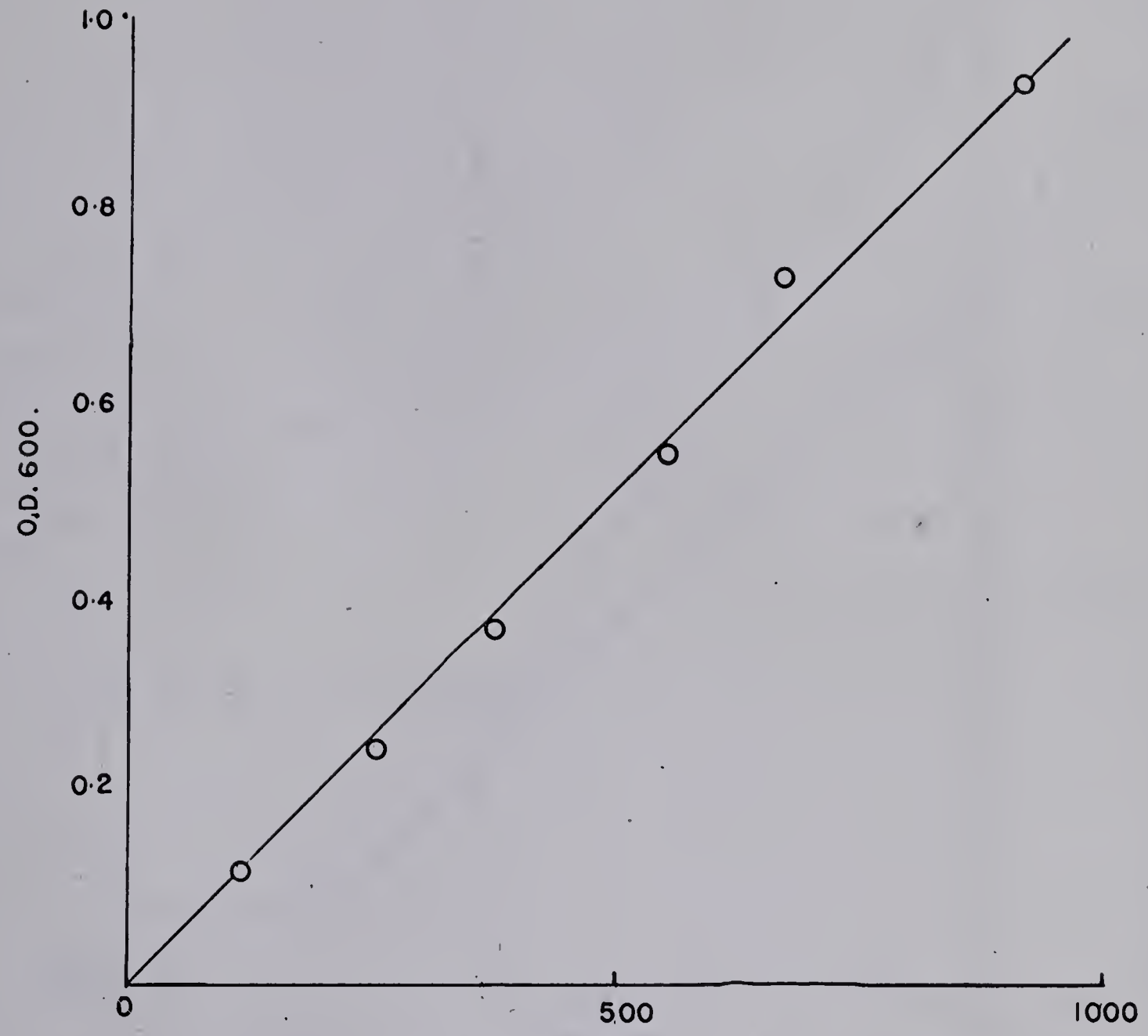
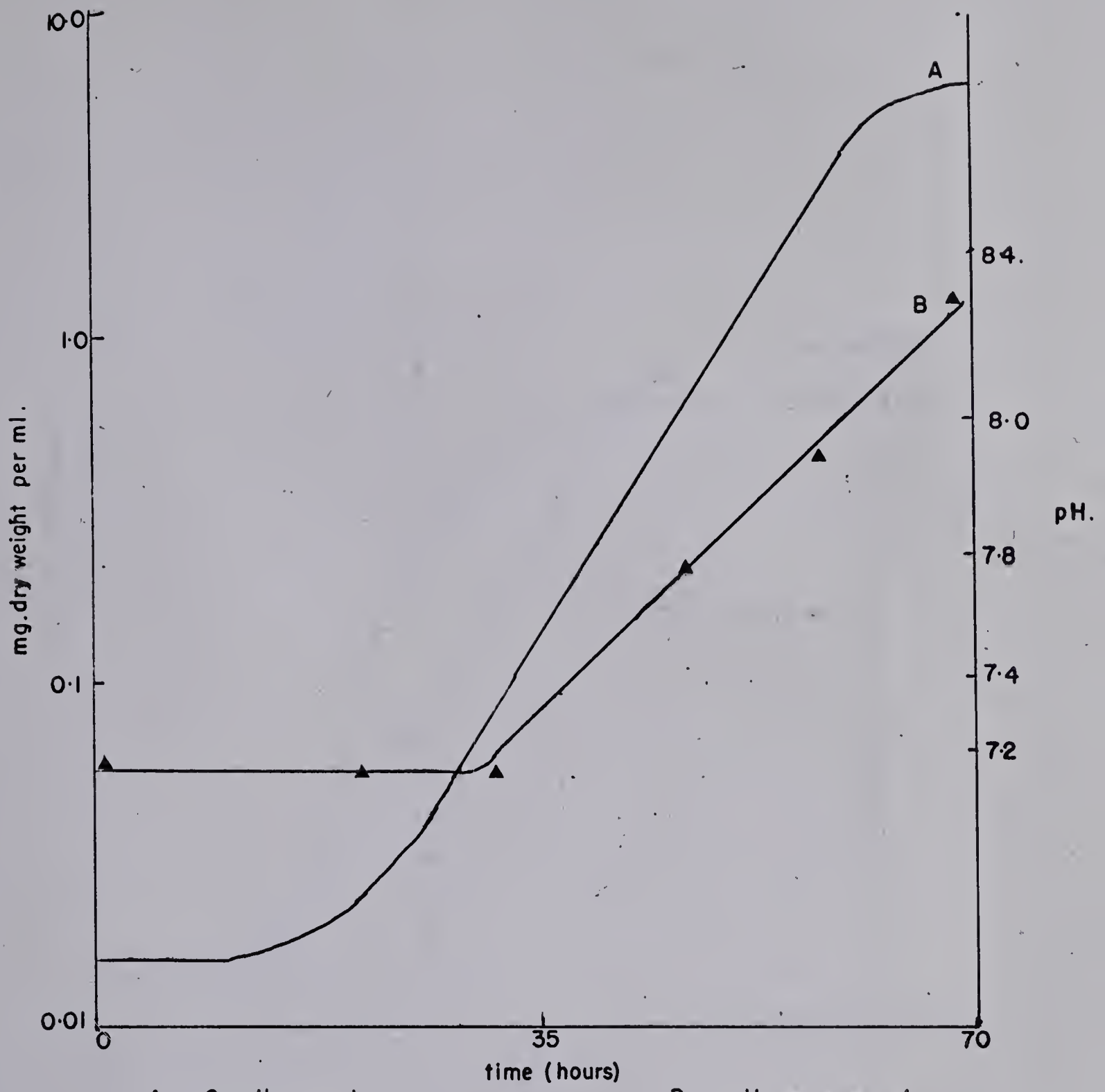


FIG.4. CALIBRATION CURVE RELATING TURBIDITY TO DRY WEIGHT OF CELLS OF ATCC 15174.



A — Growth curve : B. — pH measurement

FIG.5. CHANGES IN pH IN GSM DURING GROWTH OF ATCC 15174 AT 14° C .

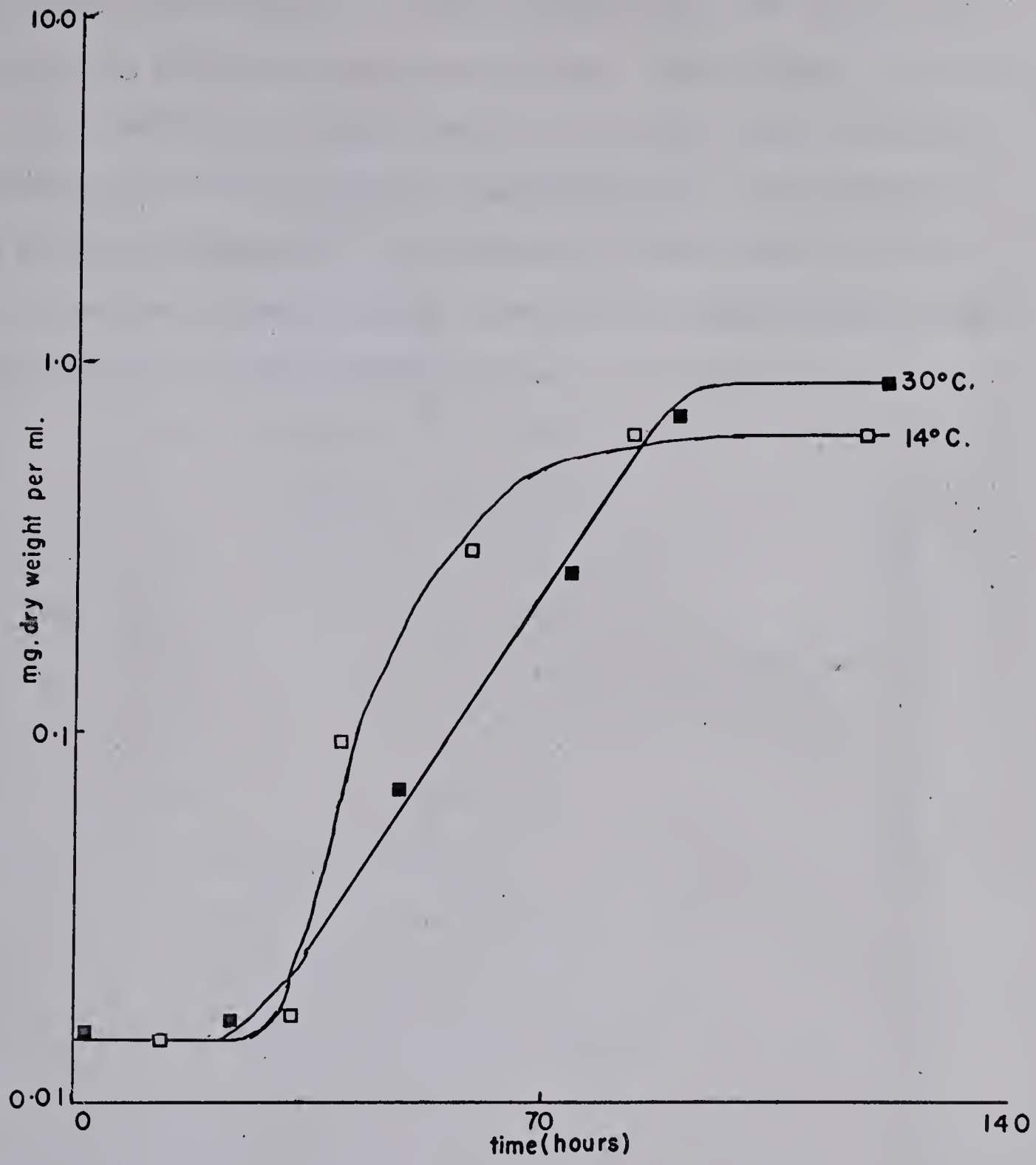


FIG. 6. GROWTH CURVES FOR 15174—RB IN GLUTAMATE SALTS MEDIUM AT 14° C and 30° C.

Following this then is a slow decline into the stationary phase. At 30°C the exponential phase lasts almost 70 hours, the cell density doubling every 13.5 hours, and the curve resembles the usual form of a growth curve. The eventual dry weight of cells/ml. is greater at 30°C, than at 14°C, but in neither case does it approach the highest dry weight concentration of the psychrophile.

The Effect of Exposure to 30°C for Varying Periods of Time on the Growth of ATCC 15174

The effect of transferring cultures of ATCC 15174, growing at 14°C in G.S.M., to 30°C for 1, 2 or 3 hours and then returning them to 14°C is shown in Figures 7, 8 and 9. Three points within the exponential phase of growth were chosen for the transfer, and it was observed that the phase of growth determined, to some extent, the response of the organism to elevated temperature. In all cases young, dilute cultures showed an immediate increase in growth rate for two hours after which the total dry weight of cells/ml. fell even after the culture had been returned to 14°C. By contrast, the denser the cell suspension on transfer, the less adjustment of the growth rate to the new temperature and the less subsequent damage to the cell as expressed by a drop in dry weight. A relationship appears to exist between the imbalance caused to growth rate at higher temperature and the amount of damage caused. The period of recovery to the original growth rate, however, appears to be proportional to the time spent at 30°C and not to the stage of growth as can be seen by comparing the three figures.

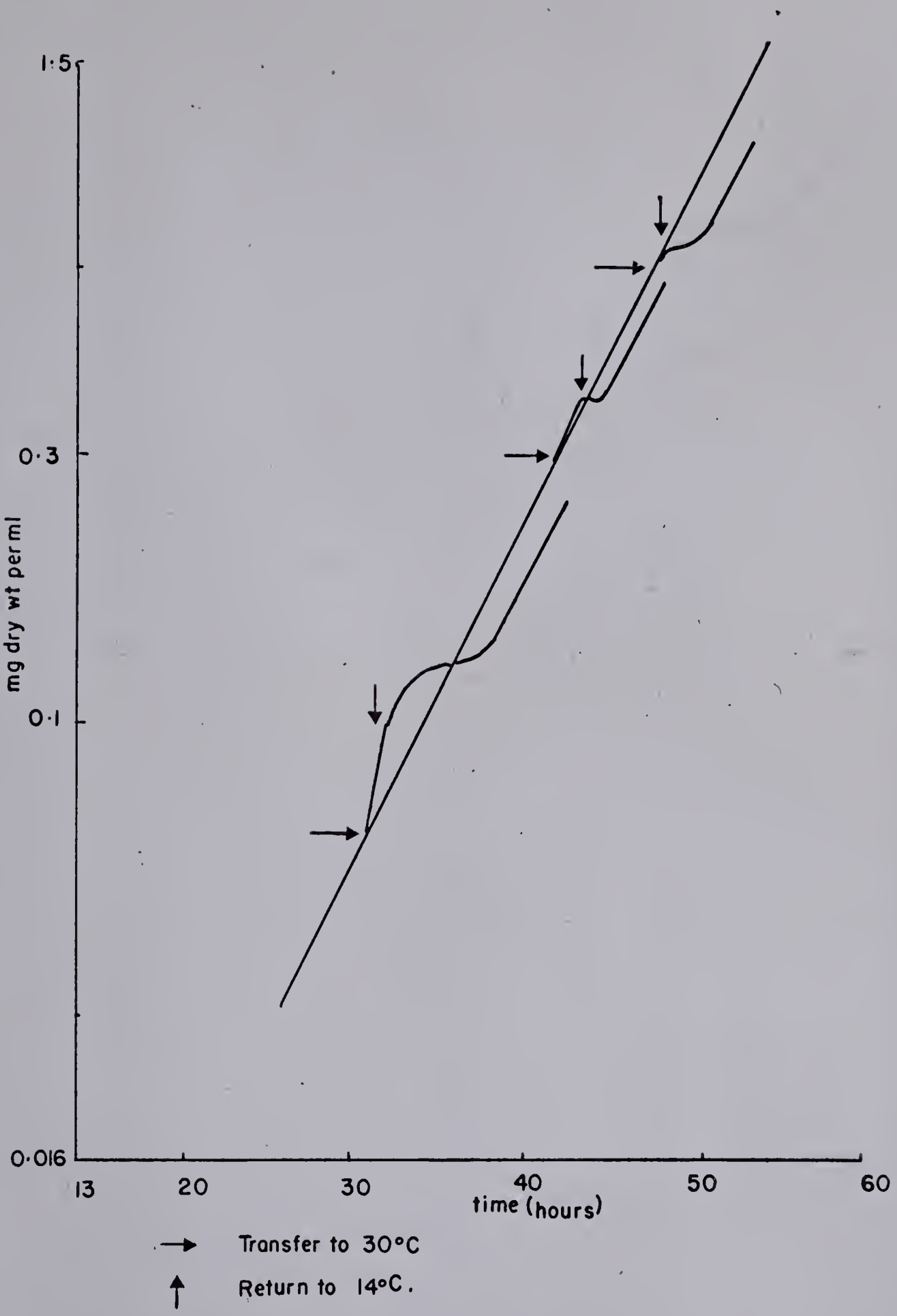


FIG.7: EFFECT OF 1 HOUR EXPOSURE AT 30°C ON SUBSEQUENT GROWTH OF ATCC 15174 IN G.S.M AT 14° C.

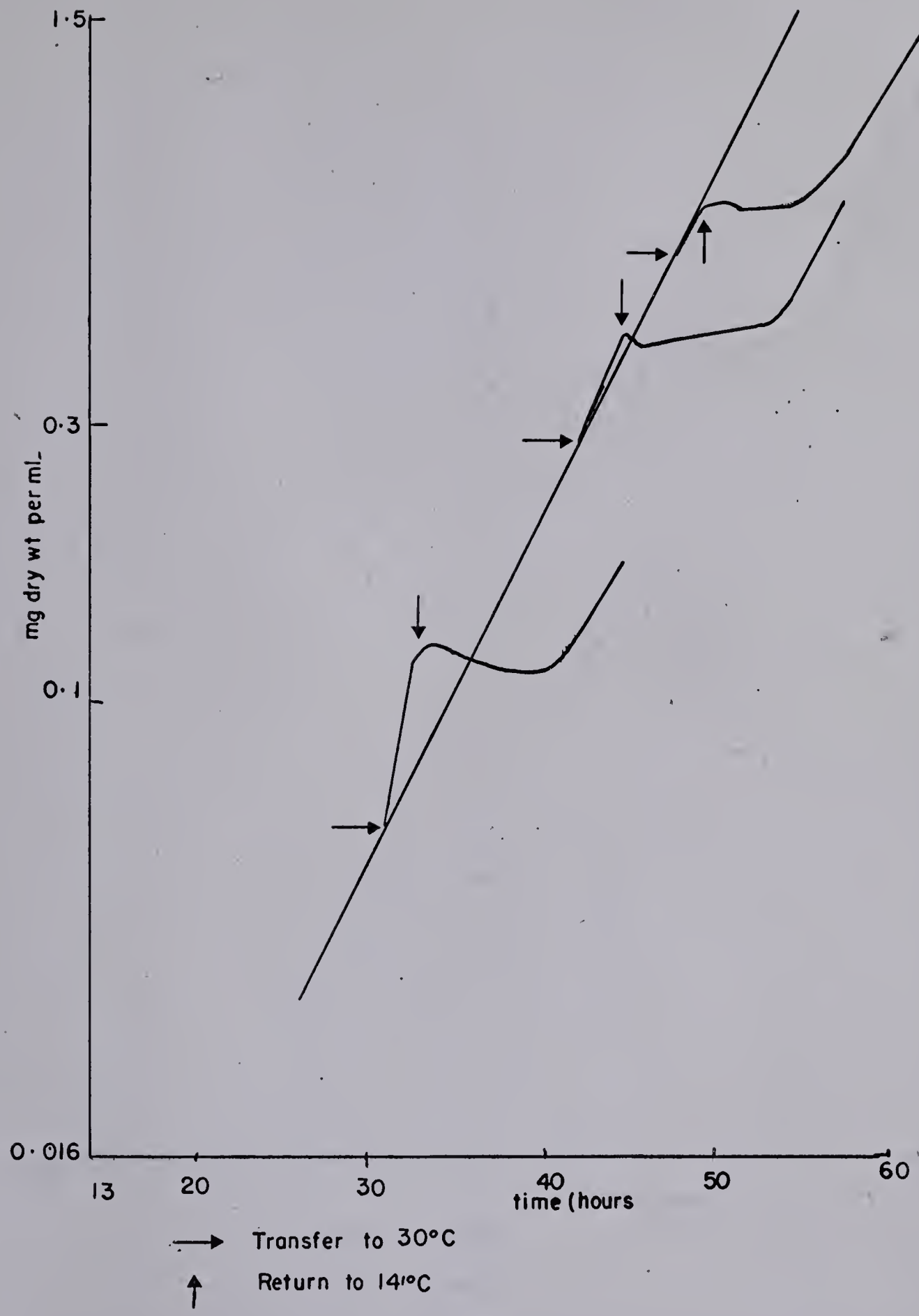
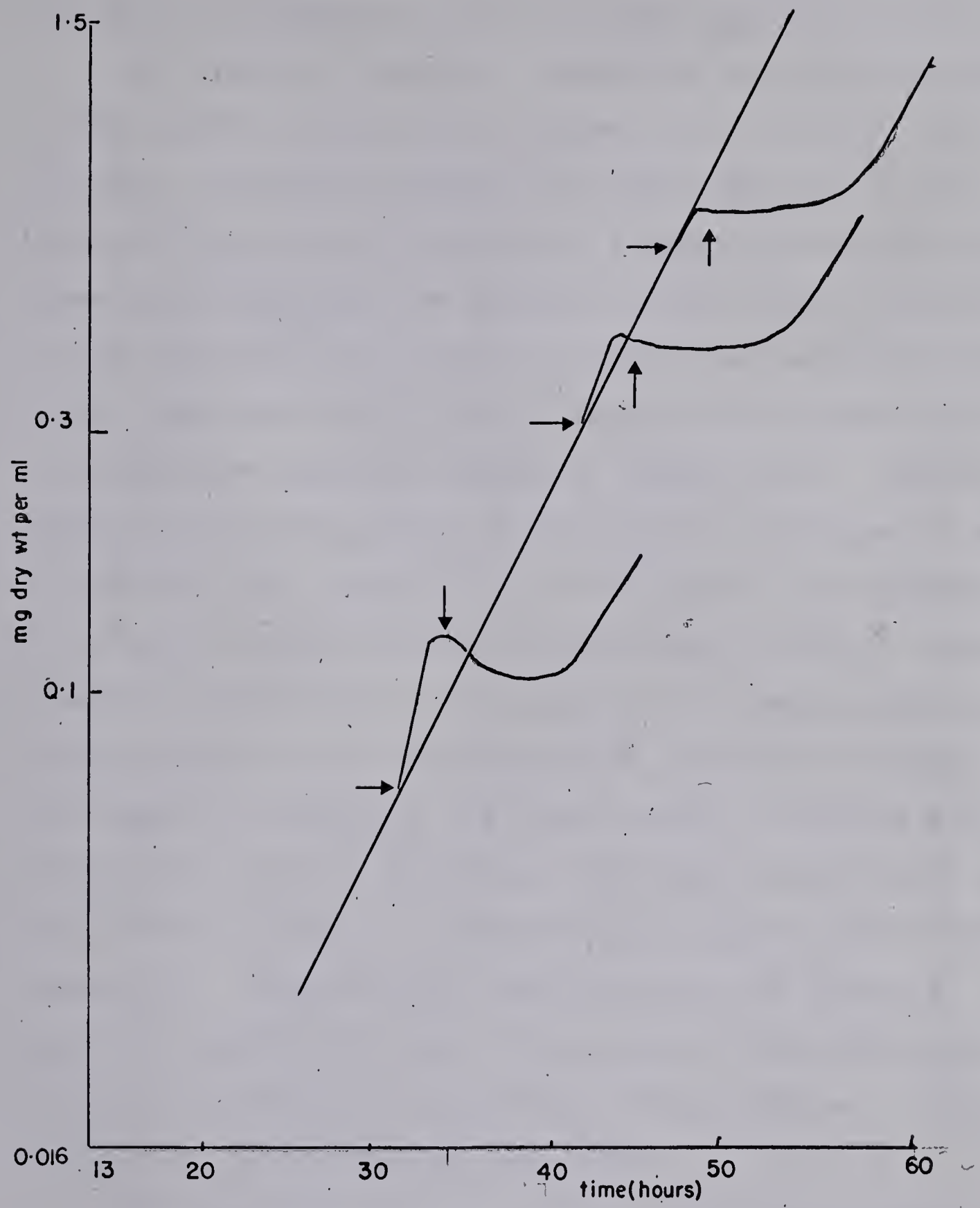


FIG.8: EFFECT OF 2 HOURS EXPOSURE AT 30°C ON SUBSEQUENT GROWTH OF ATCC 15174 IN GSM AT 14°C



→ TRANSFER TO 30°C.

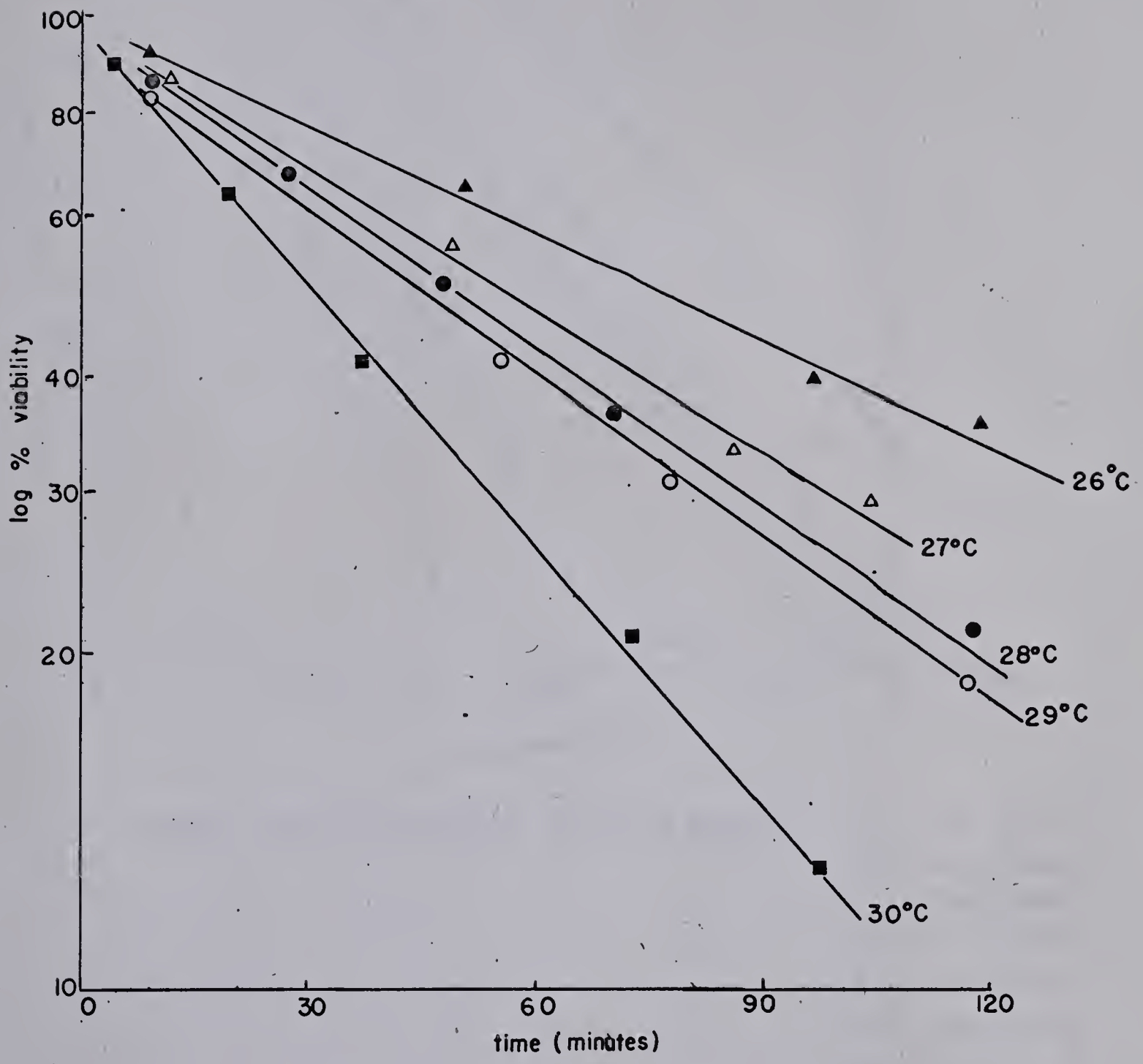
↑ RETURN TO 14°C.

FIG.9: EFFECT OF 3 HOURS EXPOSURE AT 30°C ON SUBSEQUENT GROWTH OF ATCC 15174 IN G.S.M. AT 14°C.

The Effect of Exposure to 30°C on the Viability of ATCC 15174

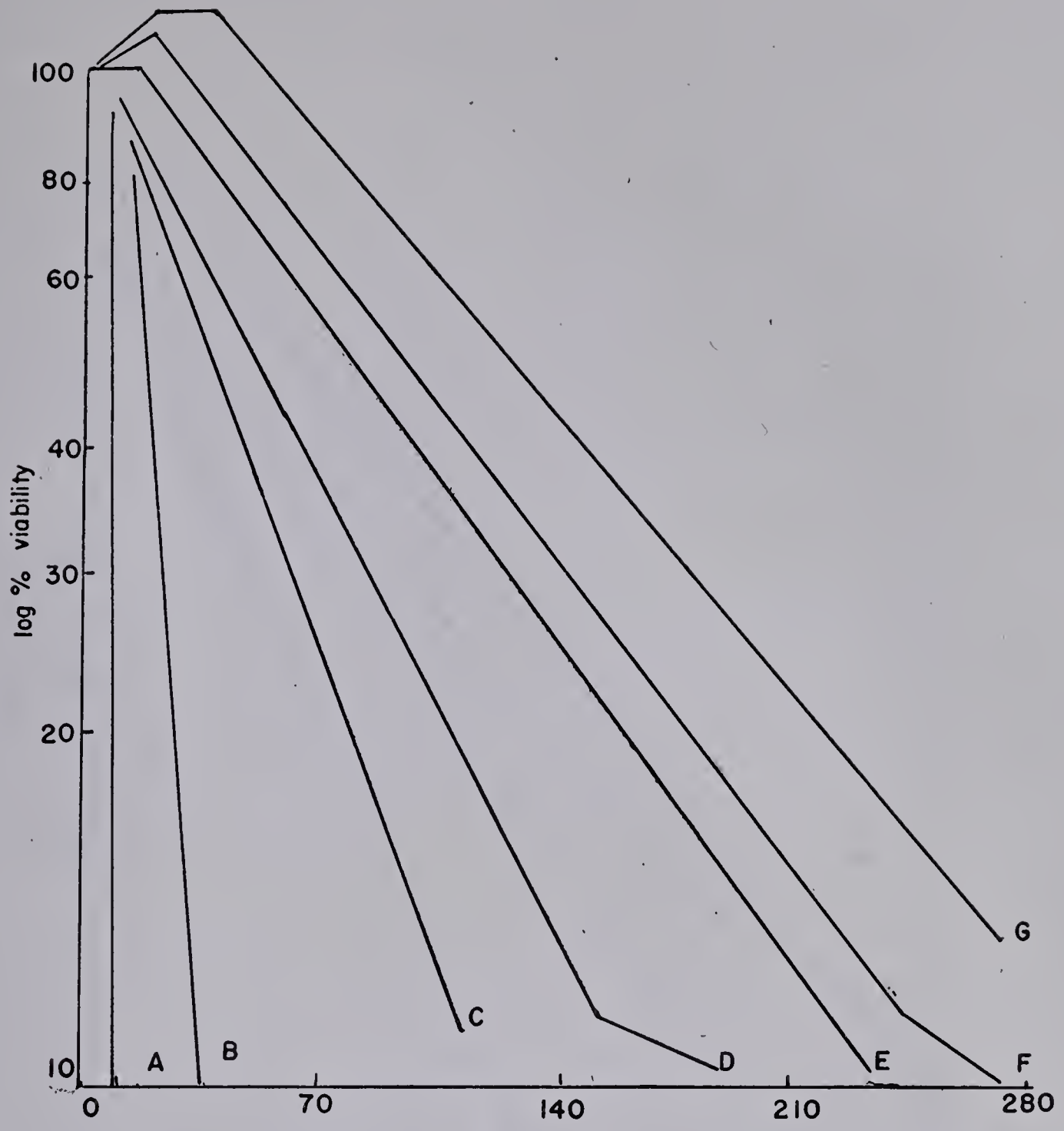
The effect of moderate temperature on the viability of ATCC 15174 is reported in Figures 10, 11 and 12. Undoubtedly incubation temperatures above 25°C have a pronounced effect on cell viability, a rapid, exponential decay commencing immediately on transfer to the higher temperature. It was shown that this loss of viability as determined by colony count was due to loss of ability to reproduce and not to temperature induced clumping of viable cells. Temperature-shocked cells subjected to mild sonication exhibited a loss of viability only after 30 to 40 minutes of this treatment. At no point during the interim period was a rise in viable numbers, attributable to breaking of the clumps, measured. It was possible by the adjustment of conditions to delay this onset of lethality, and even increase viability over a brief period, but by 35 minutes following transfer cell numbers could no longer be maintained and began to fall logarithmically. Clearly then, the heat sensitive lesion is expressed immediately and systemically following transfer, cellular reproduction being halted within minutes. Control cultures at 14°C showed no such effect.

The shape and the slope of the death curve depend upon 1) the temperature at which viability is studied, 30°C having a more rapid lethal effect than 26°C; 2) the concentration of the cells at the higher temperature. This corroborates the findings of Harrison (1960) and Postgate and Hunter (1962) that cell populations in buffer die at a slower



conditions: S.M., pH 7·5, 1×10^4 organisms originally.

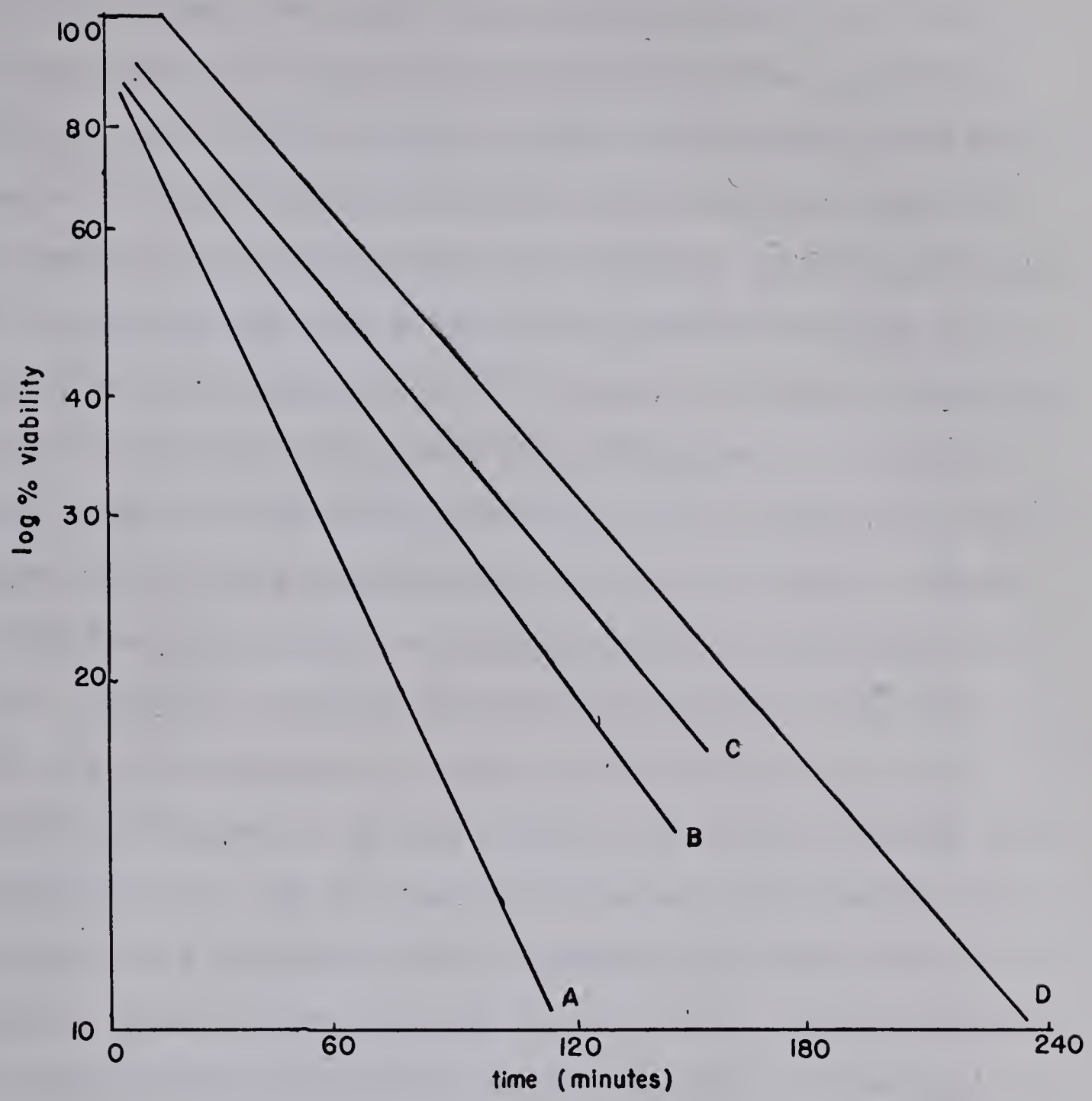
FIG.10. EFFECT OF TEMPERATURE OF INCUBATION ON DEATH RATE OF ATCC 15174.



Conditions: S.M., pH 7·5, 30 °C; Initial cell densities:

A – 170 per ml.
B – 12×10^3 per ml.
C – 35×10^4 per ml.
D – 11×10^6 per ml.
E – 88×10^6 per ml.
F – 14×10^7 per ml.
G – 67×10^8 per ml.

FIG. 11. EFFECT OF CELL CONCENTRATION ON DEATH RATE OF ATCC 15174 AT 30° C.



conditions: S.M., pH 7·5, 30°C; supplements:- A- none.
B.- 1% L-Glutamate.
C.- 3% L-Glutamate.
D.- 1% Casamino acids.

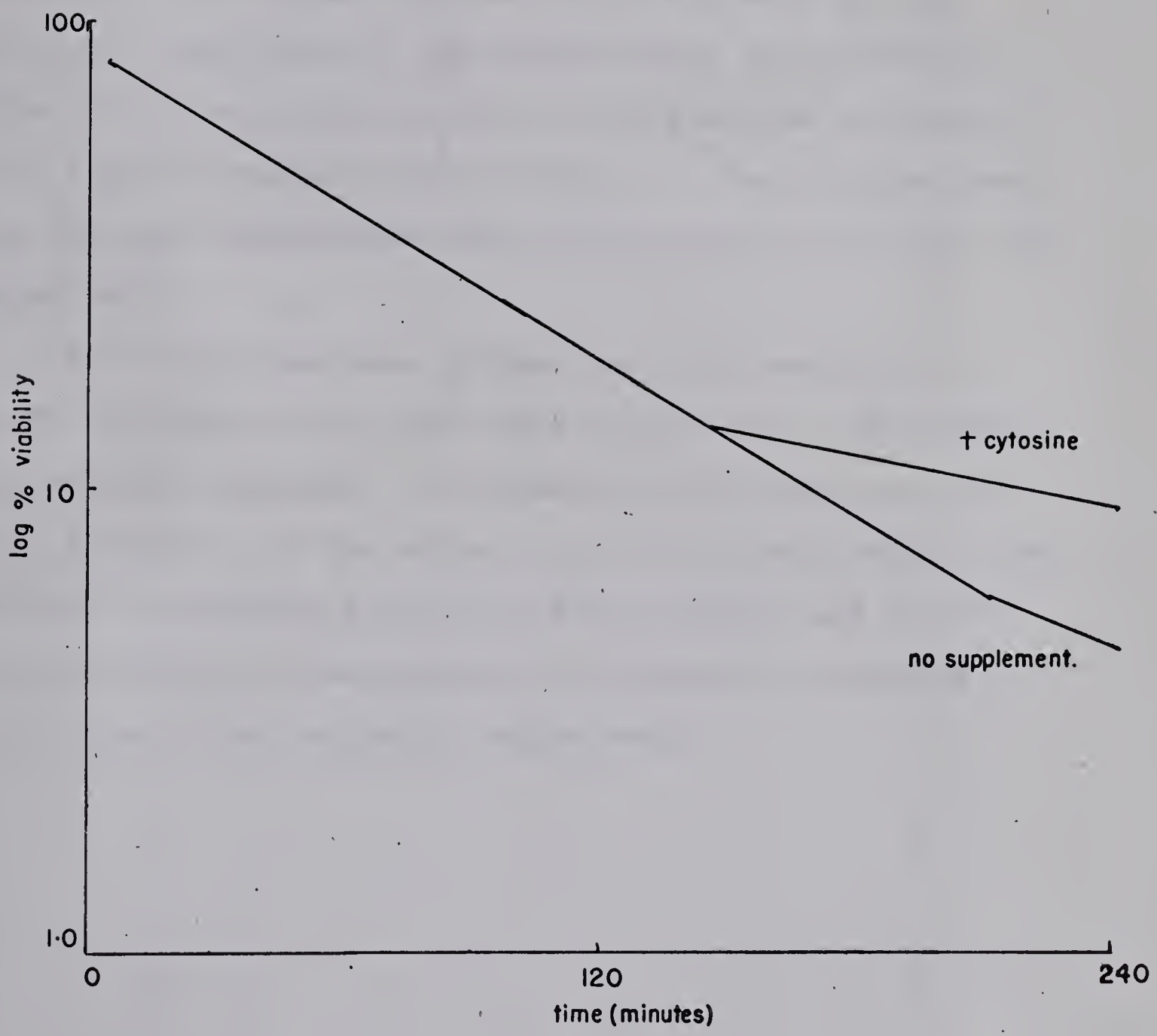
FIG. 12. EFFECT OF NUTRIENT SUPPLEMENTS ON DEATH RATE OF ATCC 15174 AT 30°C.

rate the denser the population, thus implying an interaction between the individuals comprising the population. However, this effect is short-lived and although some increase in viability may be noted, the eventual slope of the death curve is affected only slightly by concentration. It is interesting that although the greatest change in growth rate on transfer to 30°C occurs in cells in the early stages of exponential growth (viz. Figures 7, 8 and 9), these same cells show the greatest loss of viability over a given time at this temperature. Cell growth and increase in physical size might continue without cellular reproduction. Finally, although this is not directly concerned with the investigation at hand, it was noted that once viability dropped to around 2-4% in the case of dilute cell suspensions, or 10% in the case of more concentrated suspensions, the death rate fell markedly, probably due to a 'cryptic' effect (Postgate and Hunter, 1962), dead organisms providing nutrients for the survivors; 3) the presence of nutrients in the medium - the richer the supplement the lower the death rate of the organism. Knowing these factors it was possible in later experiments, when comparing loss of viability to biochemical changes within the cell, to manipulate test conditions such that a definite lag period and a definite death rate might be achieved to co-ordinate with the observed chemical results.

The Effect of Nutrient Supplementation on the Ability of ATCC 15174 to Survive at 30°C.

Hagen and Rose (1962), studying the low maximum growth temperature of a psychrophilic cryptococcus, observed that while the organism was normally non-viable at 30°C, it could be induced to grow at this temperature by prior incubation at 16°C, well within its growth range. A similar effect could be achieved by replenishing the intracellular level of keto acids, rapidly depleted at 30°C, by the addition of exogenous sources of alpha-ketoglutarate, citrate, or DL-isocitrate to the medium. The possibility of a similar situation existing when Micrococcus cryophilus was grown at 30°C was investigated by supplementation, either of the S.M. in which the exposure period was carried out, or to the recovery medium G.S.A. which was used for the viable counts. These tests showed generally a non-specific response to the presence of added nutrient, the addition of Casamino acids or Yeast extract to either medium delaying the onset of lethality for up to 35 minutes, and perhaps lessening the slope of the death curve, but certainly not promoting growth of the cells at 30°C. The substitution of synthetic mixtures of amino acids, purines, pyrimidines and vitamins, as well as carbohydrates, and metabolic intermediates could not reproduce the effect of the complex additives. The only result of note concerned later changes in the shape of the death curve when viability had fallen below 10%; this could be induced to change rate earlier by the addition of

20 micrograms/ml. cytosine to the medium, but not in the presence of the other bases (Figure 13). This point was not pursued as it appeared that any effect the cytosine did demonstrate was secondary to the feature under observation, the inability of ATCC 15174 to grow at 30°C and the sudden loss of viability of cells when transferred to that temperature.



Conditions: S.M., pH 7·5, 30°C.

FIG.13. EFFECT OF CYTOSINE ON THE DEATH CURVE OF ATCC 15174 AT 30° C.

The Effect of Nutrient Supplementation to Glutamate-Salts-Medium on the Growth of a Mesophilic Mutant Derived from ATCC 15174

Growth of 15174-RB is slow and limited in G.S.M. (Figure 6). In complex medium (T.S.B.) growth is very good and a comparison of the growth rates of the psychrophile and its mesophilic mutant in this medium is reported for a range of temperatures in Table 3. The optimum growth rate for each under these conditions occurs at 20°C and 30°C respectively.

An attempt was made by specific supplementation of G.S.M. to improve the growth rate of 15174-RB. Once again, no synthetic compounds, individually or in mixtures, promoted the growth of the mutant in G.S.M. as well as did the addition of Casamino Acids plus Yeast Extract and it was concluded that this represented a non-specific response, not related to any metabolic requirement.

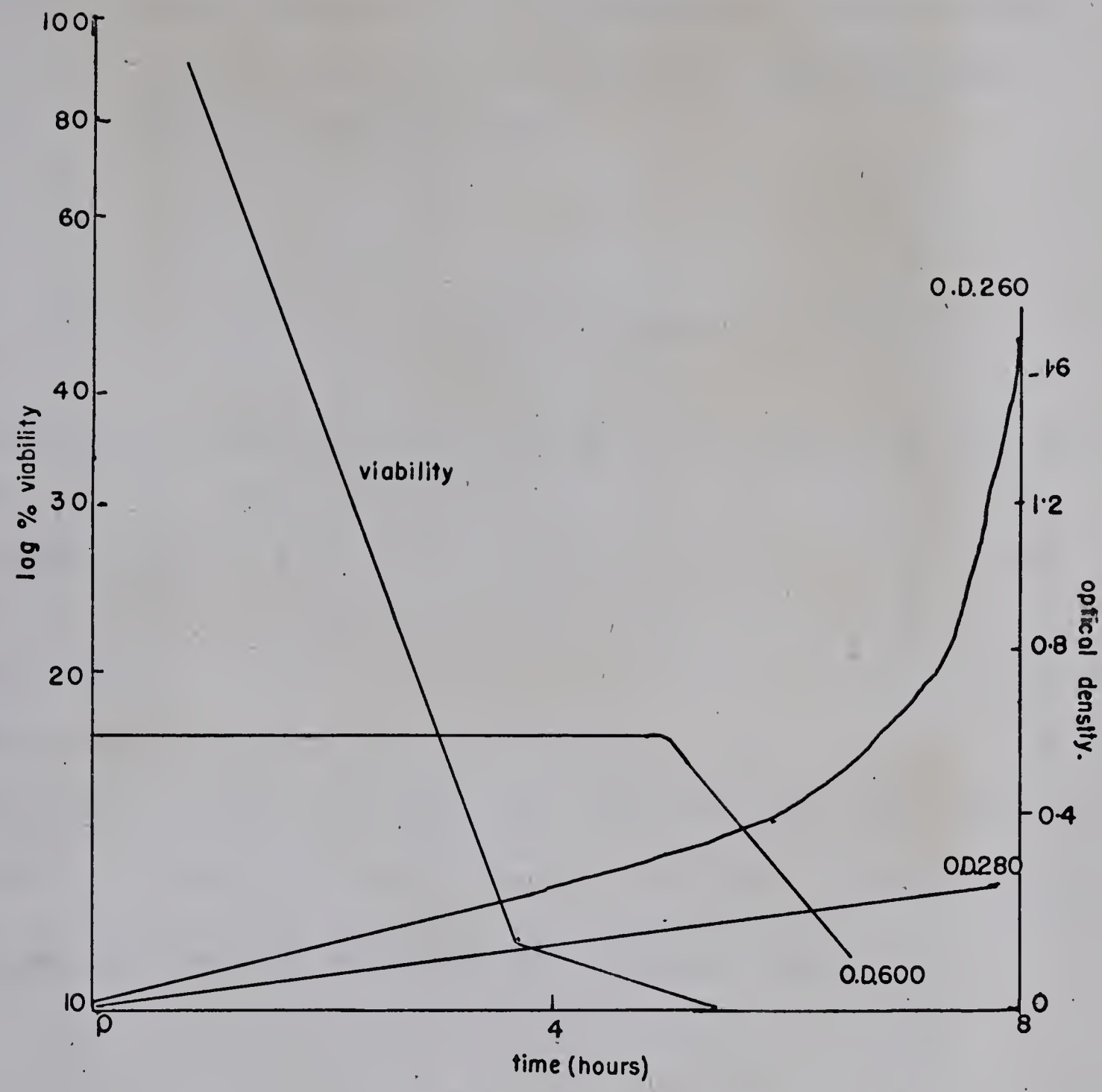
Table 3: Growth Rates (k) of ATCC 15174 and 15174-RB in T.S.B.

Temperature of Incubation °C	ATCC 15174 k.	15174-RB k.
0	0.022	0.011
3	0.045	0.016
6	0.071	0.032
9	0.139	0.064
12	0.149	0.091
15	0.180	0.106
18	0.213	0.158
20	0.255	0.277
24	0.213	0.290
27	-	0.304
30	-	0.319
32	-	0.213
34	-	0.138
36	-	-

Studies on Membrane Integrity at 30°C

Is the biological integrity of M. cryophilus retained for a time after temperature-induced death or is it lost with the onset of death? It has been proposed that changes in the liquid-crystalline nature of membrane lipids may sometimes be involved in determining the maximum temperature for growth (Luzzati and Husson, 1962; Byrne and Chapman, 1964). In another piece of work on a psychrophile a facultative strain of Vibrio marinus, Morita and Burton (1963) suggested that permeability change as well as enzyme lability was a partial reason for the maximum growth temperature of that culture. The sudden and severe effect of a temperature of 30°C on viability points to an area of cellular physiology vital to the functioning of the organism being affected. Membrane integrity is such an area and this was studied.

The relationship between loss of viability at 30°C, cell lysis and leakage of intracellular materials is shown in Figure 14 and Table 4. In a fairly heavy suspension of cells in S.M., viability falls exponentially for almost 4 hours, approximately half the cells dying in the first 90 minutes. Cell density, as measured by optical density at 600 millimicrons, remains constant for around five hours after which it falls off quite rapidly. Leakage of 260 millimicrons- and 280 millimicrons-absorbing material is not significant during the first four hours following transfer to 30°C, but becomes more apparent, at 260 millimicrons



Conditions: S.M., pH 7·5, 30°C; Viability and O.D. 600 measured on culture.

O.D.260 and OD. 280 measured on cell free supernatant.

FIG. 14. EFFECT OF EXPOSURE TO 30°C ON VIABILITY, OPTICAL DENSITY AT 600mμ, LEAKAGE OF 260 and 280mμ ABSORBING MATERIALS INTO THE SUPERNATANT.

Table 4: Leakage of Intracellular Materials From ATCC 15174 in Buffered Salts Medium at 30°C; and Comparison With Dry Weight of Cells

	Time of Incubation (Hours)									
	0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
RNA	0	2	3	6	8	10	15	18	26	40
Protein	0	0	0	0	0	0	0	2	8	10
DNA	0	0	0	0	0	0	0	0	3	5
Amino Acids	0	0	0	0	0	0	0	0	0	1.2
Keto Acids	0	0	0	0	0	0	0	0	0	0
Dry Weight	550	550	550	550	550	540	530	510	490	480

(Concentrations are expressed as micrograms/ml.)

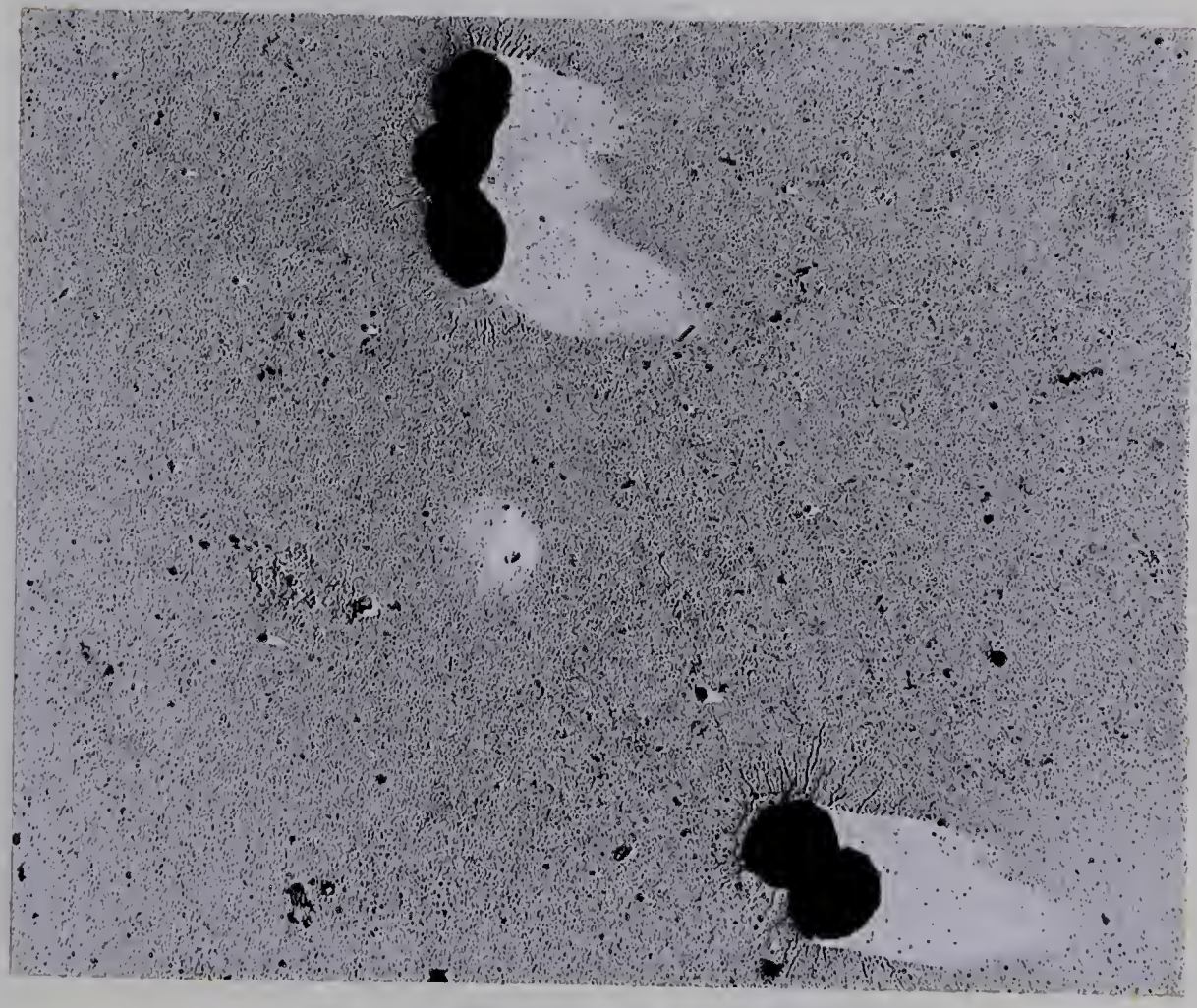
The results for all the supernatant examinations represent the difference over a control at 14°C.

especially, after 5 hours, the same time as cell lysis commences. The two curves for ultraviolet absorption in Figure 14 represent the difference over a control at 14°C. The results of chemical analyses on these supernatants (Table 4) indicates that orcinol-reacting compounds are by far the main component of the leaked material; protein is negligible and DNA, amino acids and keto-acids are just detectable, if at all, during the last few hours of the test. Again all the figures quoted represent the difference over a control at 14°C which showed little loss of viability over the same period. Comparable results for release of 260 millimicron- and 280 millimicron-absorbing compounds, were obtained when ATCC 15174 was incubated at 30°C in G.S.M. for the same period of time. These findings indicate that death of ATCC 15174 at 30°C precedes lysis, a comparable result to that observed by Hagen et al. (1964) when studying temperature-induced death and lysis in a psychrophilic Gram-negative rod.

Electron micrographs prepared from samples withdrawn during exposure of the cells to 30°C indicated no apparent signs of surface damage (Figures 15, 16). The shadow-cast preparations appear unchanged. Similarly in the phosphotungstate-fixed samples; no evidence of fine damage is available from the photographs shown. In each case the photographs presented are representative of many that were taken.

Another criterion of membrane damage at 30°C is the permeability of ATCC 15174 to dyes of the anilino-naphthalene sulphonic acid class, which fluoresce only when they

A

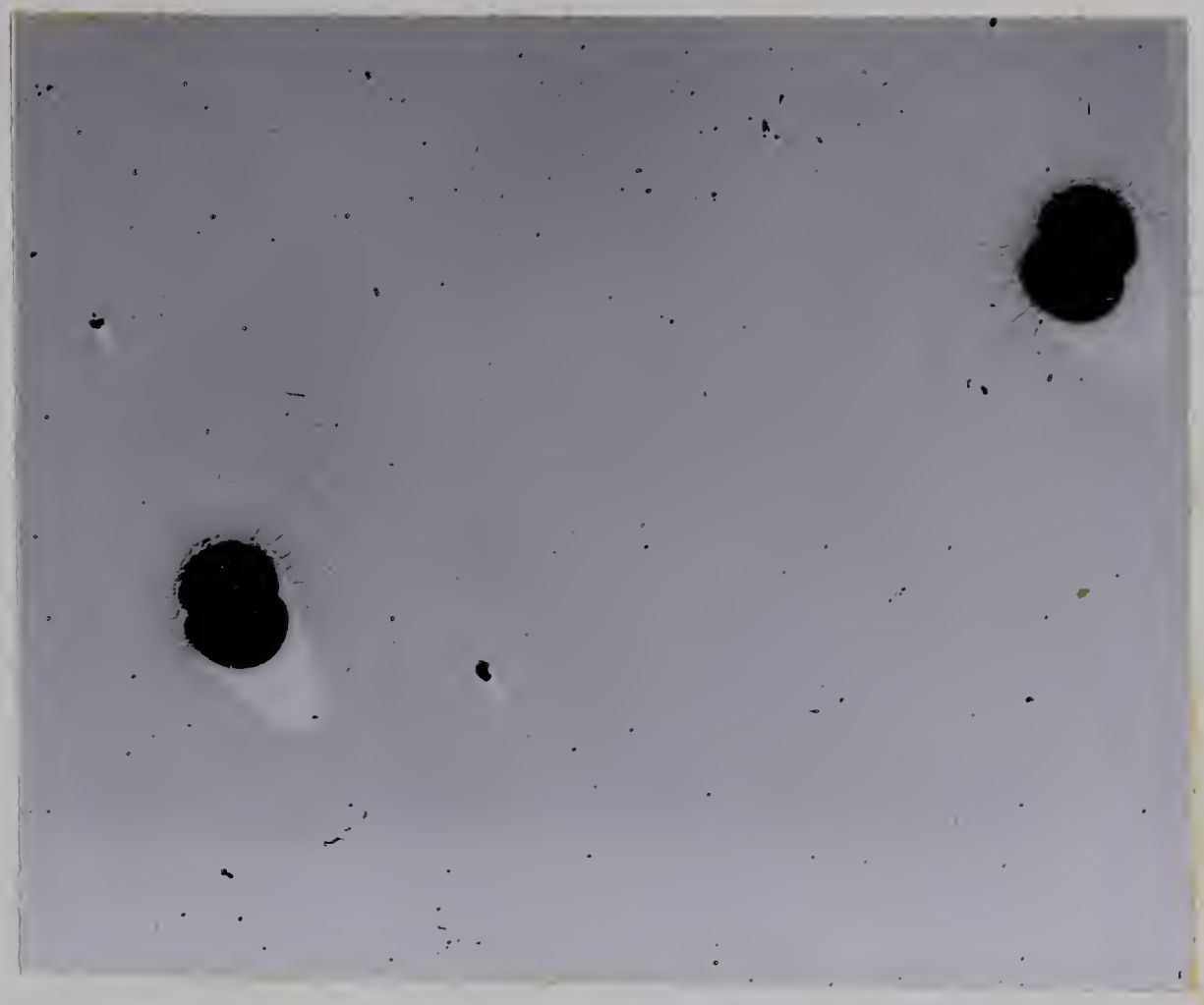


B

A - 0 Hours: B - 2 Hours

Figure 15: Shadow-cast Electron Micrographs of ATCC 15174 Suspended in S.M. 30°C. (12,000 x Magnification).

Figure 15 continued....

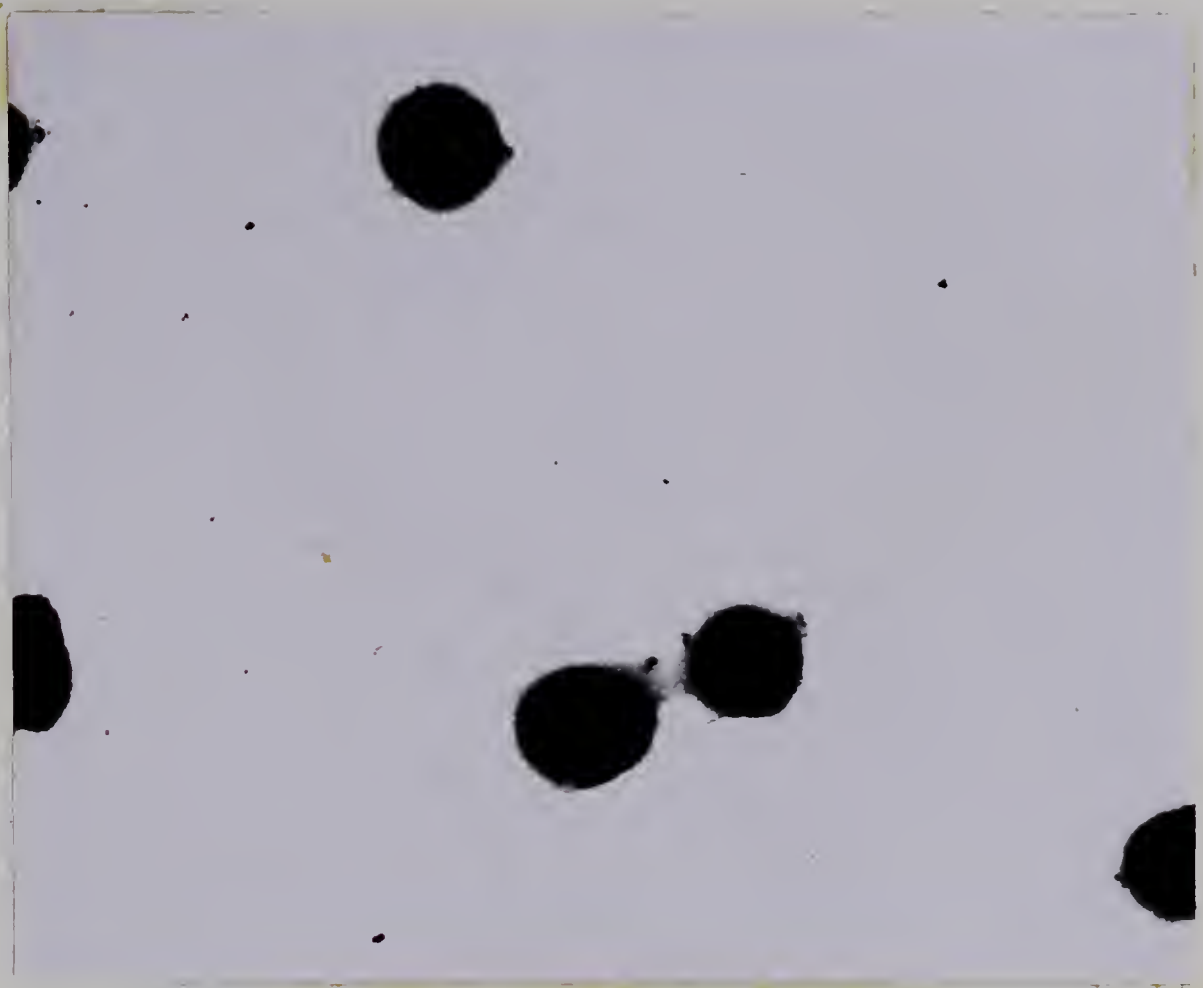


C

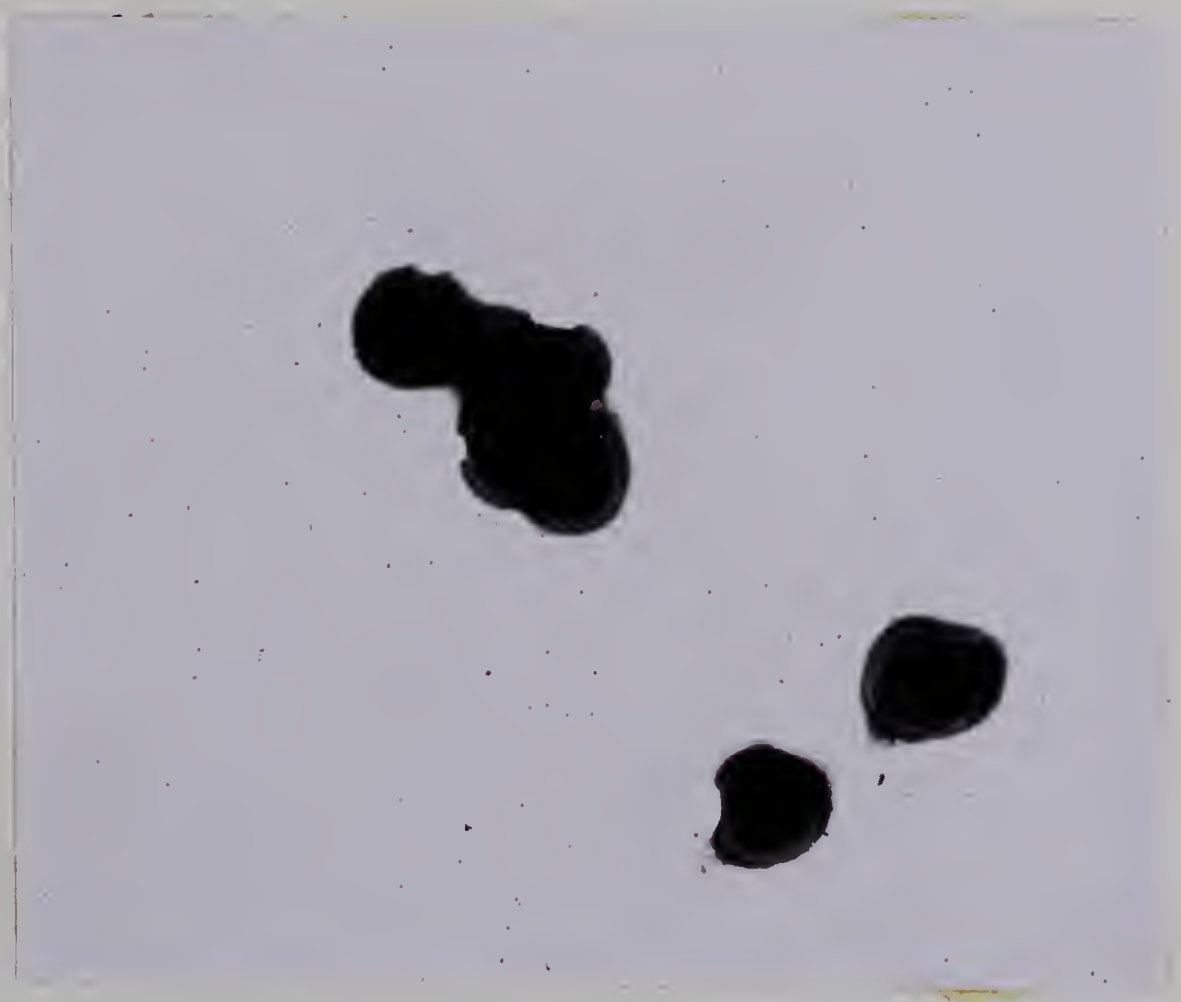
D

C - 4 Hours; D - 8 Hours

Figure 15: Continued



A

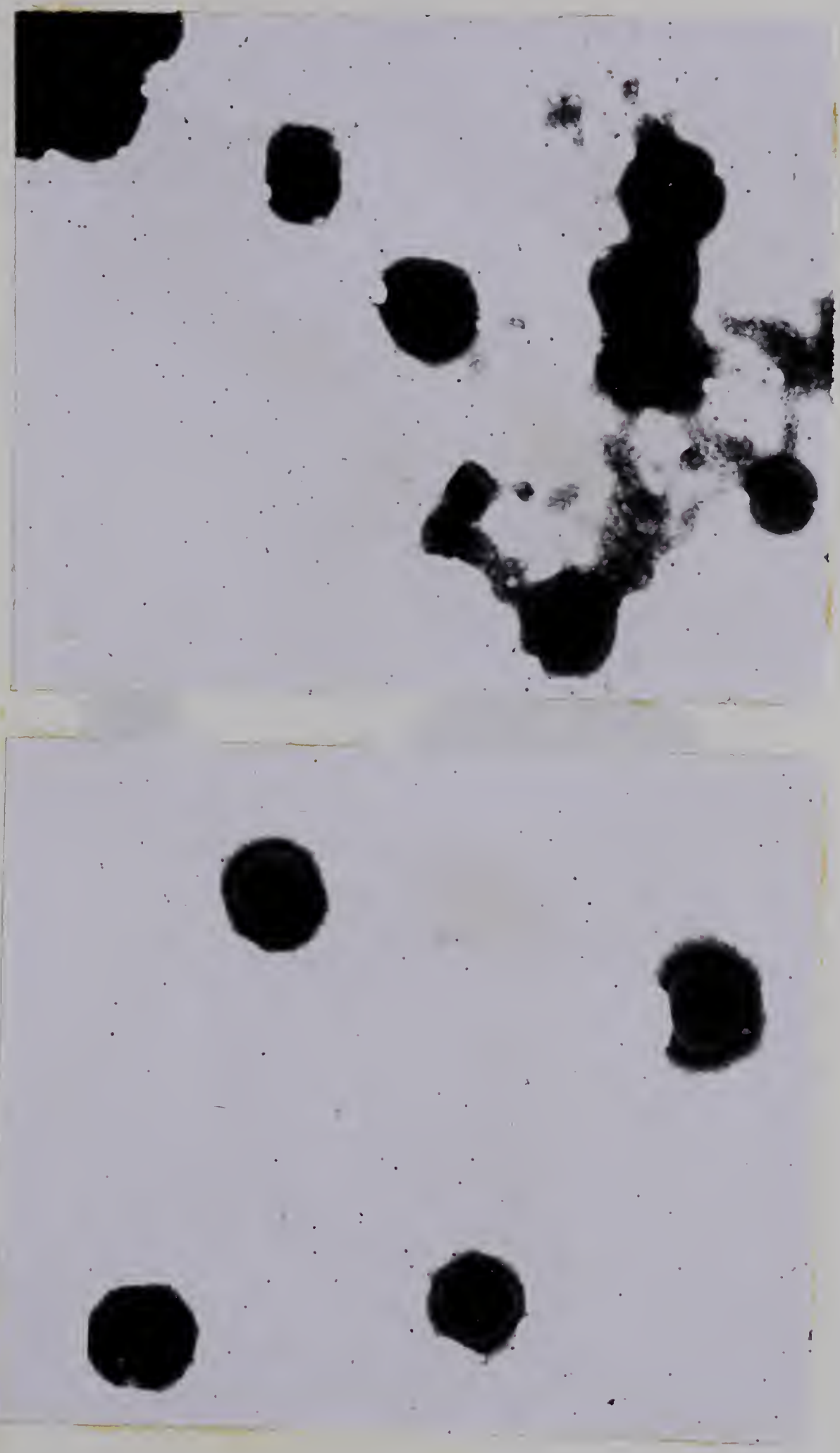


B

A - 0 Hours; B - 2 Hours

Figure 16: Electron Micrographs of Phosphotungstate Preparations of ATCC 15174 Suspended in S.M. at 30°C. (12,000 x Magnification).

Figure 16 Continued...



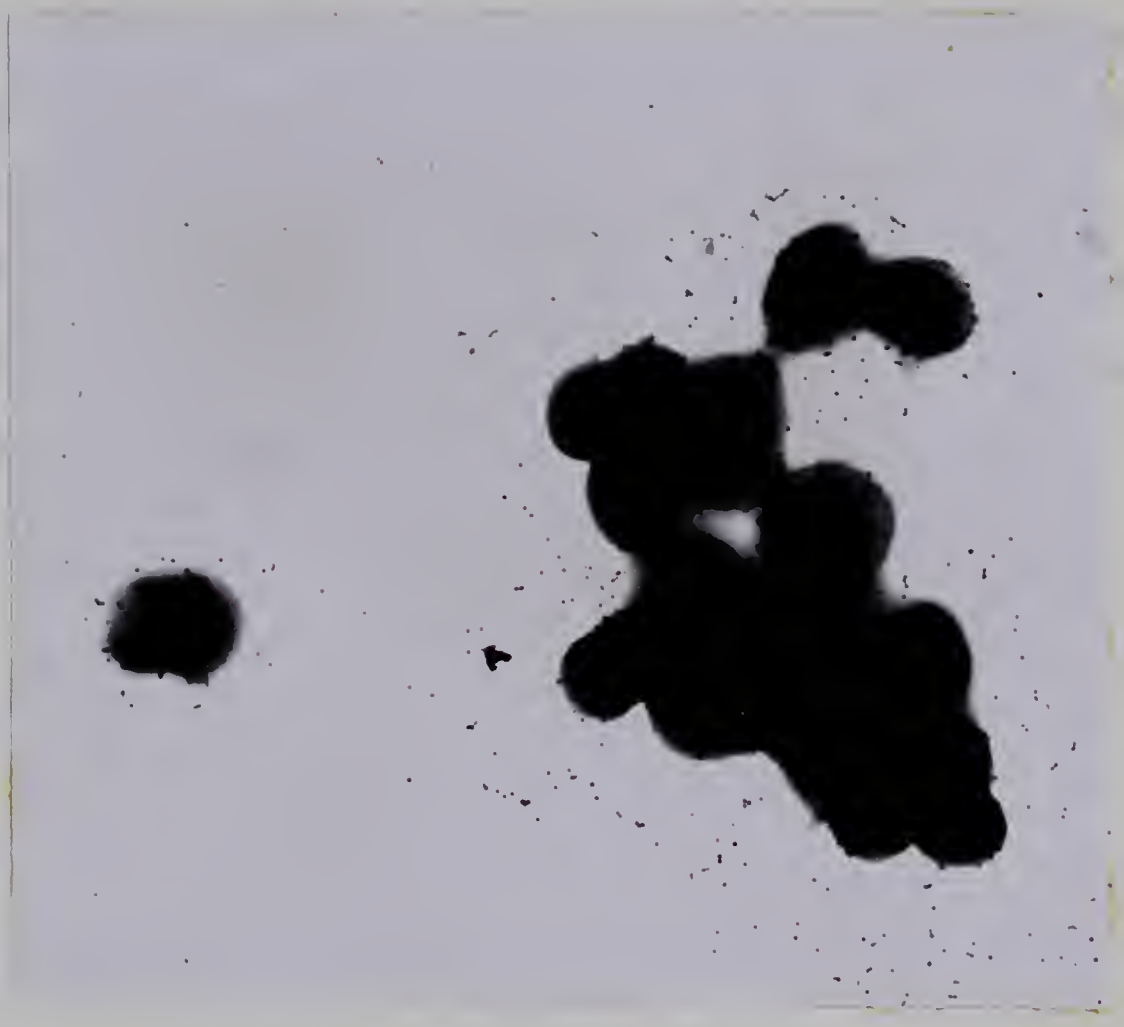
C

D

C - 4 Hours; D - 6 Hours

Figure 16: Continued

Figure 16 continued...



E

E - 8 Hours

Figure 16: Continued

have penetrated the cell and conjugated with the protein therein. The rate of membrane damage thus detected, does not mimic the rate of viability decline (Figure 17), but appears once again to be a secondary phenomenon subsequent to the death of the cells at 30°C. A control culture at 14°C showed little loss of viability over this period and only 2% fluorescence by 8 hours.

Thus, at least something of the biological integrity of the bacterial cell is retained for a time after death resulting from exposure to elevated temperatures.

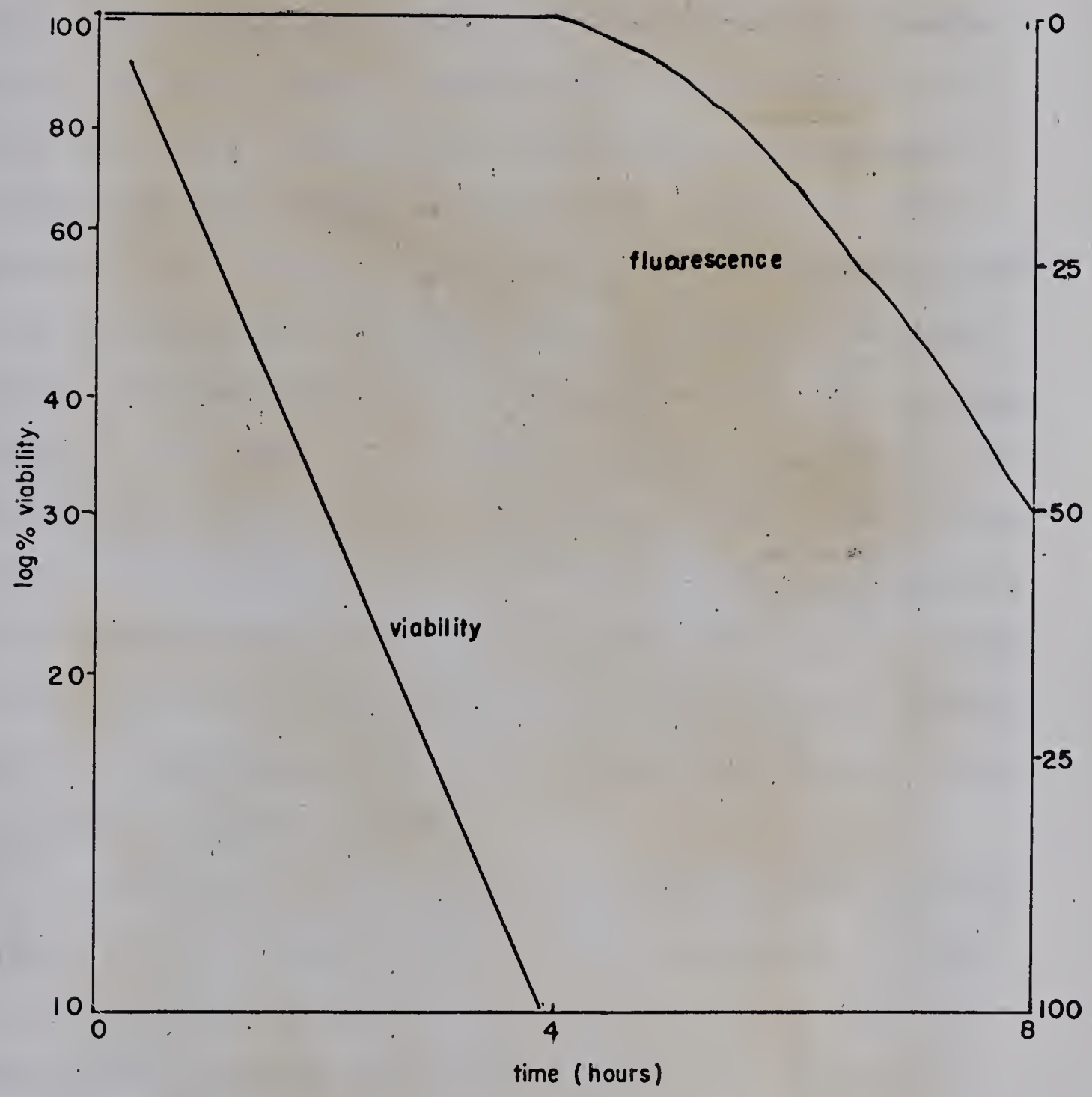


FIG. 17. EFFECT OF EXPOSURE TO 30°C ON MEMBRANE AS EXPRESSED BY PENETRATION OF FLUOR-ESCENT DYE INTO CELL.

Studies on Intermediary Metabolism

Enzyme denaturation is usually assumed to be a major factor in determining the maximum temperature for growth. Evison and Rose (1965), Upadhyay and Stokes (1963) and Burton and Morita (1963) have all reported the presence of heat-sensitive enzymes in the T.C.A. cycles of their psychrophiles, although these were not related specifically to the determination of temperature for growth. Considering the relative importance of the Krebs' Cycle to *M. cryophilus* and its inability to obtain energy by glycolysis or fermentation, might not the loss of activity of one or more of the cycle enzymes be proposed as a plausible explanation of the drastic effect of moderate temperature on the growth of this cell? If the inactivation halted a vital function like energy production, then the consequences of this lesion would ramify through the cell.

A comparison of the levels of intracellular reserves of amino acids and keto acids in cells incubated in buffer at the normal growth temperature, or exposed to moderate temperature, revealed differences (Table 5). Over the 2-hour test period the total amino acid content of the cells rose very slightly at 14°C while at 30°C there was an initial drop and then an accumulation in the pool. Keto acids remained unchanged in the control, but fell steadily in concentration on exposure to 30°C, the relative amounts of the two components of the pool separated by electrophoresis - alpha-ketoglutarate and pyruvate - remaining almost constant.

Chapter 4: Thermodynamics of Cycling

Energy Conservation in Humans

Cycling in Thermodynamic Equilibrium

Steiger and Abbe (1953). Changing wet-bulb and Dalton and Hardie (1957) have all measured the dynamics of heat-equilibrium changes in the 2 to 3 hours of exercise preceding it, although there are limited exceptions to the maintenance or temporary increase of the body temperature during the period. Therefore, the body is in equilibrium with the environment and the quantity of heat produced (internal-plus) equals the quantity of heat dissipated (internal-minus). Under this condition of equilibrium the cycle ergometer is measuring all of the energy expended by the muscles. If the ergometer setting is altered, the body temperature will rise or fall until equilibrium is again established.

A comparison of the ratios of internal energy produced to energy measured by the ergometer is given in Table 4.1. The ratio is the total energy produced by the body divided by the energy measured by the ergometer. Values of 4.0 (Asmussen and Nielsen, 1955) and 4.1 (Nielsen, 1938) have been used to estimate the total energy produced by the body from the ergometer measurement. Values of 3.8 (Christensen, 1931) and 3.9 (Christensen and Hansen, 1939) have been used to estimate the total energy produced by the body from the ergometer measurement

Table 5: Effect of Temperature of Incubation on Endogenous Amino Acid and Keto Acid Reserves

Hours Incubation at 14°C	Total Amino Acids (micrograms/mg. dry weight of cells)	Total Keto Acids (micrograms/mg. dry weight of cells)
0	30.6	10.2
0.5	30.8	*
1.0	31.4	10.5
2.0	31.6	10.4
Hours Incubation at 30°C		
0	30.6	10.2
0.5	25.1	10.8
1.0	30.0	9.1
2.0	32.0	7.0

* Samples lost.

Conditions: S.M., pH 7.5; 35% loss of viability over 2 hours in cells at 30°C; no loss of viability in control.

Table 3: Effect of temperature on inoculation at exponential phase with 4g/l folic acid treatment

hours incubation at 18°C	Local within Nevada infectogenicity. def. Variation of Initial	Local Area to be determined/ any variation on colony
0	[illegible]	[illegible]
0.5	[illegible]	-
1.0	[illegible]	[illegible]
2.0	[illegible]	[illegible]

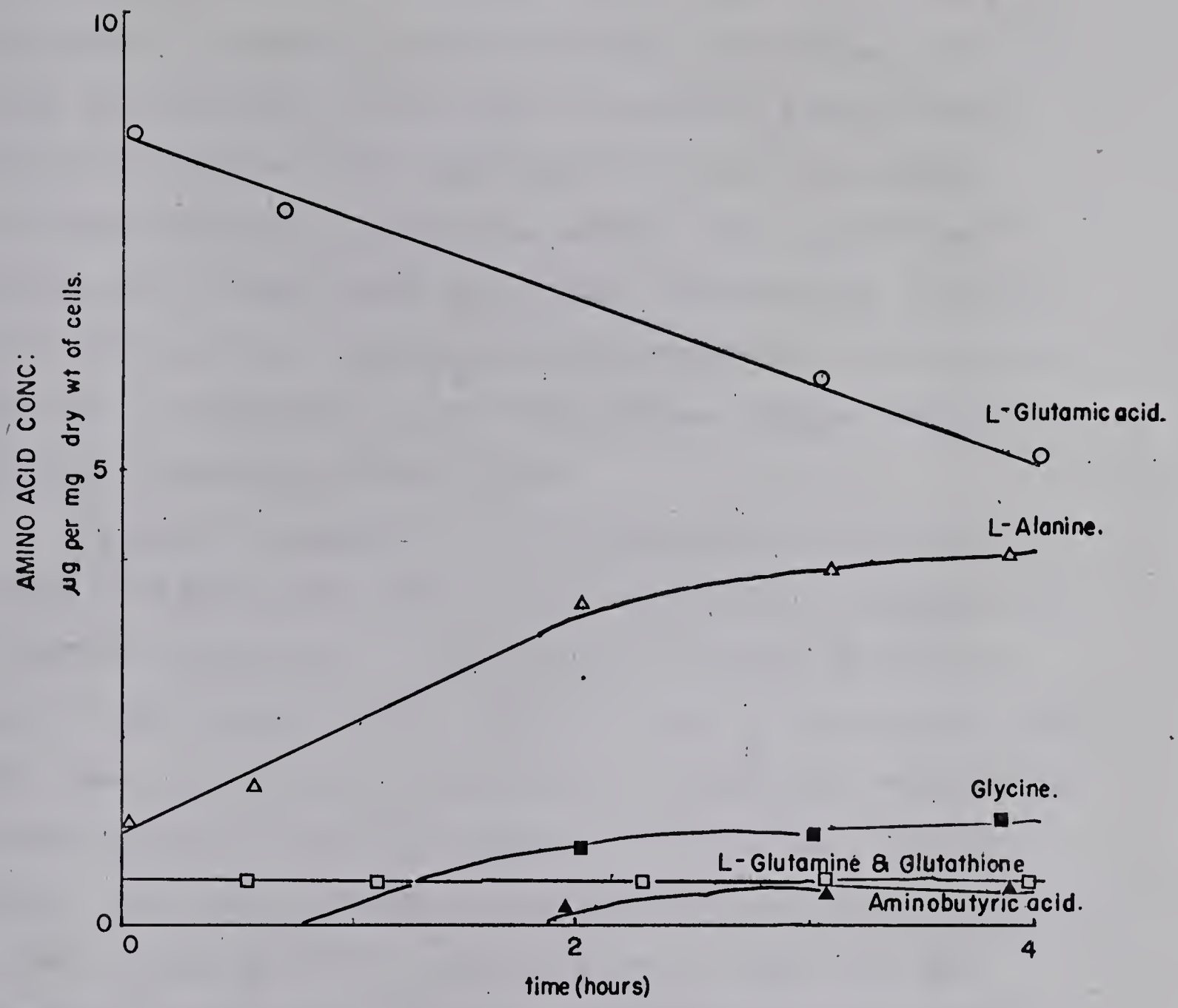
hours incubation at 30°C		
0	[illegible]	[illegible]
0.5	[illegible]	[illegible]
1.0	[illegible]	[illegible]
2.0	[illegible]	[illegible]

Conclusions:

Conclusions: [illegible] were less in remaining cases in order to [illegible] of viability in general

The data shown represent the average of 3 readings taken at each sampling period and the possibility of loss due to cellular leakage was discounted by the negative results obtained when portions of concentrated cell-free supernatants were examined for these compounds.

The examination of the changes in the amino acid content of 30°C cells was extended over a 4 hour period, this time an effort being made to characterize and quantitate the components of the pool (Figure 18). Glutamic acid, alanine, glutamine and glutathione were detected in the cell at zero time, glutamic acid comprising the bulk of the pool. Glutathione was detected in the oxidised form if care were taken to spot the filter paper quickly and commence chromatography immediately. If the dried sample was left for some time before separation then usually the two forms of glutathione, oxidised and reduced, appeared in the final analysis. As incubation of the cells at 30°C proceeds, distinct patterns of change are shown. First, the reserve of glutamic acid is metabolized steadily through the duration of the test, while glutamine and glutathione remained constant. Alanine, however, increased rapidly in concentration from the moment of transfer until it reached a maximum after 2 hours. Glycine and aminobutyric acid were detected one and two hours respectively after transfer, and each rose in concentration for the subsequent hour, soon after which a plateau was reached. (It was not possible to decide from the chromotogram whether the latter was gamma- or alpha-aminobutyrate). Clearly, glutamate is being metabolised rapidly

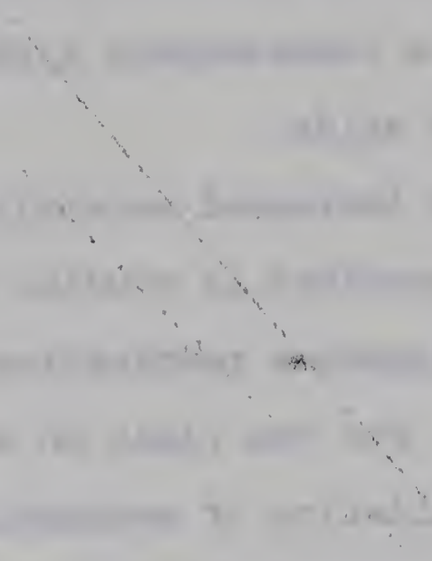


Conditions: S.M., pH 7·5, 30°C.; 80% loss of viability over 4hrs.

FIG 18: THE EFFECT OF EXPOSURE TO 30°C ON THE AMINO ACID POOL OF ATCC 15174.

on transfer to 30°C while other amino acids are being synthesized. A possible explanation for this circumstance is that glutamic acid enters into a series of transamination reactions with suitable keto acids to yield alpha-ketoglutarate plus the above amino acids. The sequence of appearance of each amino acid might indicate that a particular keto acid is transaminated preferentially then another species is catalyzed by the transaminase system. Hence the drop in intracellular keto acids.

Further evidence for increased metabolic turnover at moderate temperature was provided by studies on oxygen consumption (Figure 19). Endogenous respiration at 30°C is much higher than at 14°C. For the first 20 minutes the oxygen taken up due to the oxidation of endogenous reserves is almost equal to the total uptake in the presence of glutamate. The level of oxygen consumed reaches a plateau between 1 and 2 hours at 30°C, presumably most of the cellular reserves having been oxidised. Endogenous respiration at 14°C, however, is maintained at a constant rate of less than 5 microlitres per hour. When total oxygen uptake by cells at the two temperatures of incubation is compared in the presence of glutamate as the oxidizable exogenous substrate, they are quite similar. On the basis of these results it would appear that, although the cells of ATCC 15174 were dying at 30°C, their respiratory activities continued. When the respiratory rates of this organism at different temperatures were compared (Figure 20), it became obvious that



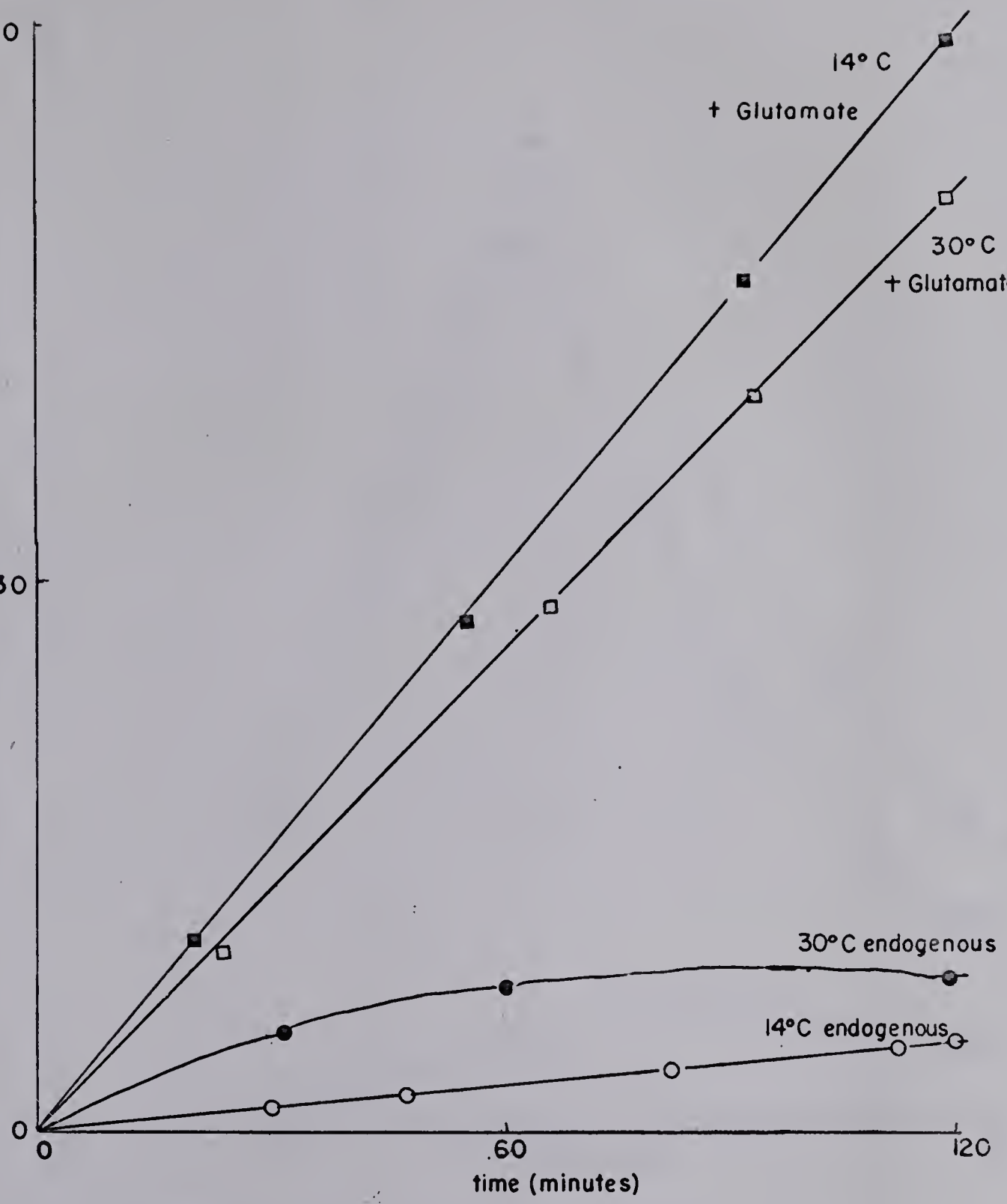
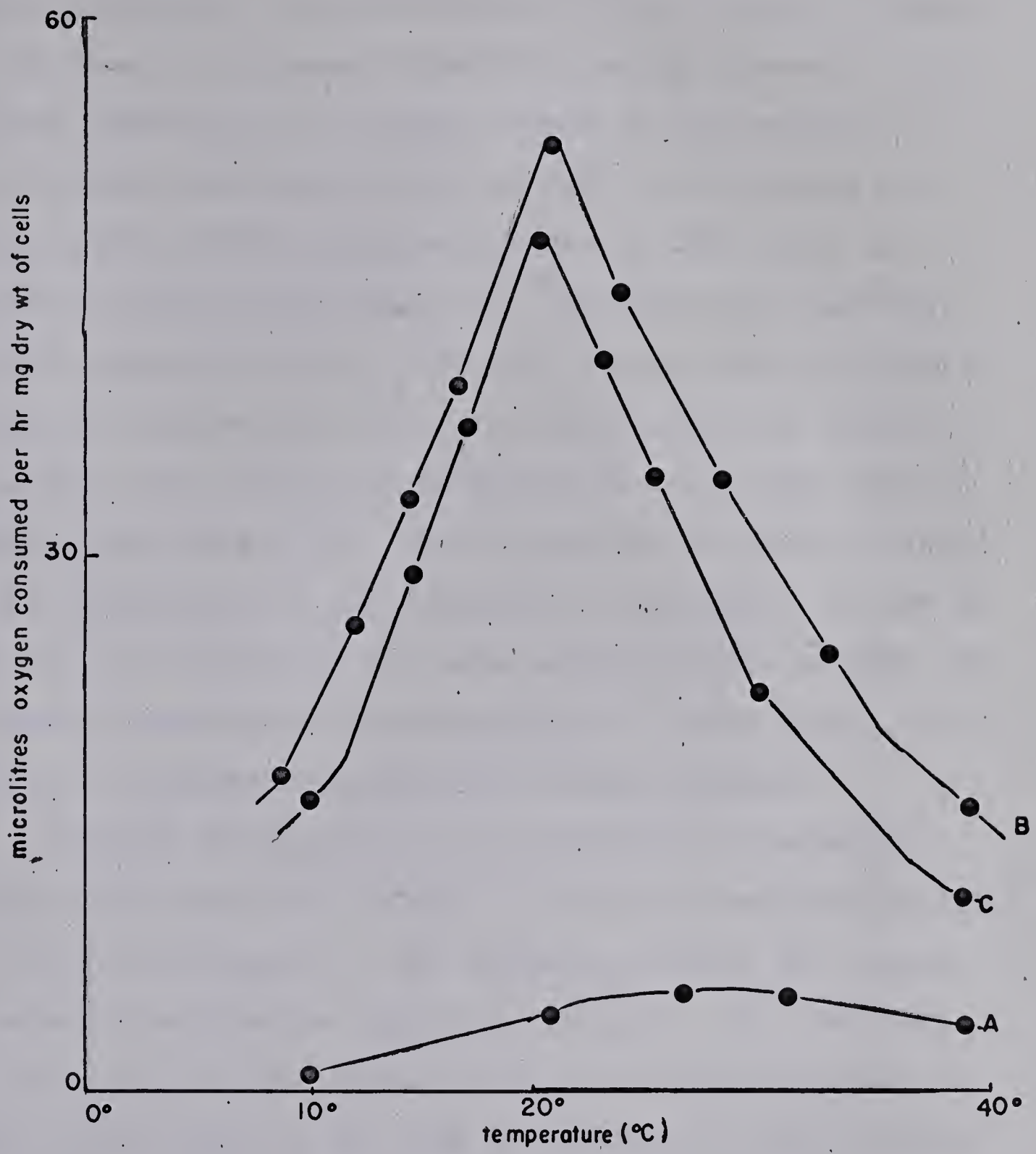


FIG. 19: OXYGEN CONSUMPTION OF ATCC 15174 AT 14°C and 30°C IN THE PRESENCE and ABSENCE OF GLUTAMIC ACID.



Legend: Curve A.— Endogenous respiration.

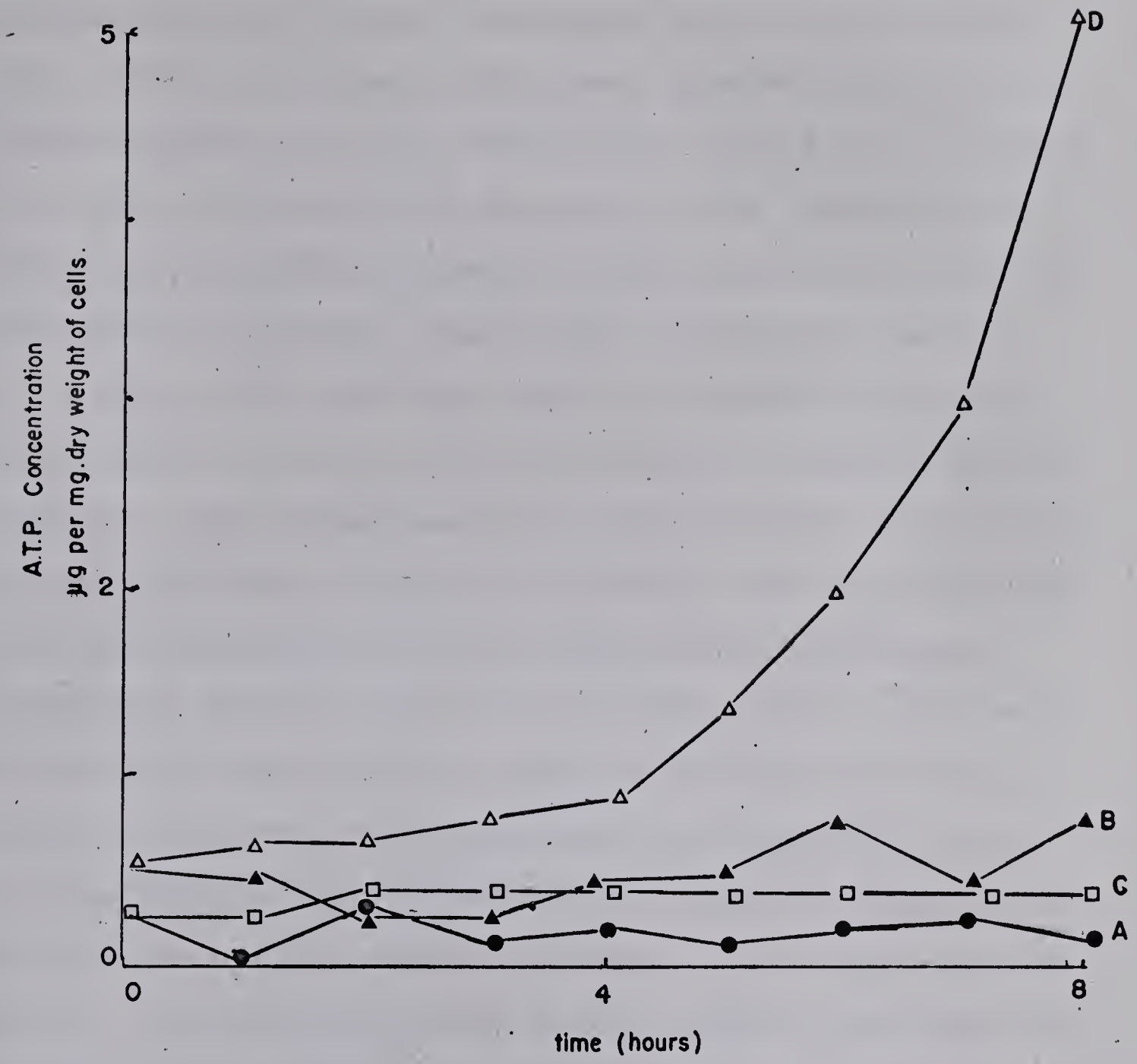
Curve B.— Respiration in presence of oxidizable substrate (Glutamate)

Curve C.— Exogenous respiration (B-A).

FIG.20: RESPIRATION RATES OF ATCC 15174 AT DIFFERENT TEMPERATURES IN THE PRESENCE OF AN OXIDIZABLE SUBSTRATE.

oxygen consumption continued above 20°C, no drastic change in rate due to increased temperature being observed. The optimum temperature for oxygen uptake in the presence of an oxidizable substrate occurs at 20°C, this finding correlating with studies on growth rates of ATCC 15174 at different temperatures (Table 3). Endogenously, however, maximum respiration is at 26°C, one degree above the maximum growth temperature and it is notable that the curve drops much more quickly at temperatures below this than at temperatures above 26°C. When correction is made for endogenous respiration in the oxidation of glutamate, it can be seen that the curve has the same maximum uptake at 20°C, but at higher temperatures the proportion of total uptake attributable to endogenous respiration becomes greater.

Although respiration is not seriously affected by temperatures above 25°C, there is still the possibility that the related phenomenon of ATP synthesis during the course of terminal respiration might be affected. That this was not the case is illustrated in Figure 21 which relates the total ATP in cells to the time of exposure to 14°C or 30°C in the presence and absence of glutamate. The ATP content of cells at 14°C in S.M. or G.S.M. fluctuates slightly during the course of the test but remains fairly constant, the level in the cells grown in a nutrient medium increasing towards the end of the test when cell numbers were increasing. The curve showing the effect of 30°C in S.M. rises over the first two hours, when viability drops by 50%,



Legend: Curve A - S.M., 14°C.
Curve B - G.S.M., 14°C.
Curve C - S.M., 30°C.
Curve D - G.S.M., 30°C.

FIG. 21: EFFECT OF TEMPERATURE OF INCUBATION ON A.T.P. CONTENT OF ATCC 15174.

and then remains constant throughout the remainder of the test. In the presence of glutamate, however, there is a steady increase in total cellular ATP content for the first four hours as viability is dropping to 20%, after which there is a tremendous increase in ATP concentration for the remainder of the test. It is clear, therefore, that: 1) cells of ATCC 15174 are capable of synthesizing ATP while exposed to 30°C, and will continue to synthesize this compound - even though the cells are non-viable - if an oxidizable substrate is present (a further examination of respiration on glutamate at 30°C showed there was oxygen consumption over the eight-hour period); and 2) when cells of ATCC 15174 are exposed to 30°C in buffer, there is a rise in ATP concentration over the first two hours, correlating with the data recorded for endogenous respiration, and the level of ATP remains constant for the rest of the 8 hours. Either synthesis is exactly equal to utilization or, more likely, synthesis and utilization have both halted. In summary then, terminal respiration as measured by oxygen consumption or ATP synthesis, is not affected by moderate temperature.

Final evidence bearing on this hypothesis is presented in the results of a survey of the activities of the enzymes of intermediary metabolism, particularly those of the T.C.A. cycle and those associated with glutamate metabolism. These enzymes were assayed in crude extract, no attempt being made at purification, or at separation of membrane-containing and

soluble fractions of the extracts. The levels of T.C.A. cycle enzymes vary during the growth cycle (Wimpenny, 1966) and the experiments were carried out with cultures harvested at a standard extinction during the logarithmic part of the growth cycle. Extracts were prepared from cells which had been incubated throughout at 14°C and from cells which had been incubated at 30°C, long enough to lead to a 40% loss of viability, just prior to fractionation. The first extracts were assayed for activity at 14°C and 30°C and the latter at 30°C, with the aim of detecting, if present, a specific heat-sensitive enzyme. The results are recorded in Table 6 as specific activities - micromoles substrate consumed per hour per mg. of protein in cell extracts - and are the average of three assays.

All enzymes of the T.C.A. cycle were detected, although at varying levels, due probably to the method of preparation of the extracts and the previous conditions of growth. The presence of low activities of pyruvic dehydrogenase is explainable by the fact that there is no glycolytic pathway producing pyruvate as an intermediate. Aconitase and fumarase in E. coli have been reported to be rapidly turned over and often lost during extraction, while the initial level on minimal medium-grown cells is quite low (Wimpenny, 1966).

No deleterious effect of temperature on these enzymes is noted. Generally, activities at 14°C and 30°C are comparable (with the exception of succinic dehydrogenase and

Table 6: The Activities of Enzymes in ATCC 15174 Grown in G.S.M.

Enzyme	Temp.of incubation	Temp.of Assay	Specific Activity (micromoles substrate transformed/mg.protein/hour)
Citrate synthase	14°	14°	5.0
	14°	30°	4.8
	30°	14°	5.0
Aconitate hydratase	14°	14°	0.97
	14°	30°	1.0
	30°	14°	1.1
Isocitric dehydrogenase	14°	14°	3.2
	14°	30°	4.7
	30°	14°	3.6
alpha-ketoglutaric dehydrogenase	14°	14°	6.4
	14°	30°	7.1
	30°	14°	6.4
Succinic dehydrogenase	14°	14°	30.1
	14°	30°	45.0
	30°	14°	34.0
Fumarase	14°	14°	1.0
	14°	30°	1.1
	30°	14°	1.1
Malic dehydrogenase	14°	14°	34.1
	14°	30°	38.0
	30°	14°	32.6
Pyruvic dehydrogenase	14°	14°	1.4
	14°	30°	1.3
	30°	14°	1.5

Table 6 continued...

Table 6: (Continued)

Enzyme	Temp.of incubation	Temp.of Assay	Specific Activity (micromoles substrate transformed/mg.protein/hour)
Isocitratase	14°	14°	0
	14°	30°	0
	30°	14°	0
Malic synthetase	14°	14°	0
	14°	30°	0
	30°	14°	0
Glutamic dehydrogenase($NADH.H^+$-linked)	14°	14°	0.20
	14°	30°	0.24
	30°	14°	0.21
Glutamic decarboxylase	14°	14°	1.8
	14°	30°	2.1
	30°	14°	1.8
Glutamic-oxaloacetic-transaminase	14°	14°	2.4
	14°	30°	3.9
	30°	14°	2.4
Glutamic-pyruvic-transaminase	14°	14°	1.7
	14°	30°	1.6
	30°	14°	2.0
Aspartase	14°	14°	49
	14°	30°	58
	30°	14°	42.5
Glutamic dehydrogenase($NADH.H^+$-linked)	14°	14°	0.06
	14°	30°	0.07
	30°	14°	0.08

isocitric dehydrogenase where activity increases with temperature), consistent with the results of the respiratory studies. Even exposure of the cells to 30°C, does not appear to affect these activities.

The glyoxylate cycle enzymes, isocitratase and malic synthetase were not detected. As reported, ATCC 15174 can grow in acetate plus inorganic nitrogen, but if this organism is capable of synthesising these cycle enzymes, synthesis must be repressed by growth on glutamate (Kornberg, 1965).

A variety of enzymes related to glutamate metabolism were found; none of these were temperature-sensitive. The main catalytic route whereby glutamic acid enters the T.C.A. cycle appears to be transamination. This confirms the earlier suggestion made after studying replacements for glutamate in the defined medium (Table 2) and the type of amino acids accummulated on incubation at 30°C (Figure 18). Only two transminases were assayed but possibly more were present. Glutamic dehydrogenase was detected, in low levels, but only in the synthetic direction; under the conditions of the test, and also following slight changes in pH and substrate concentrations, no catabolic activity was detected. Activity in the presence of reduced $NADP^+$ was 30% of the activity when reduced NAD^+ was present as the co-enzyme. Another catabolic pathway, decarboxylation of glutamic to gamma-amino butyric acid, was shown to exist in this organism. Previously, Halpern and Umbarger (1961) and Vender et al.(1965) reported that the acquisition of the ability

by mutants of E. coli to utilize glutamic acid as sole carbon source appeared to be related to loss of glutamic decarboxylase activity.

Finally, considerable levels of the enzyme aspartase were detected. This enzyme possibly has a significant role in the metabolism of M. cryophilus grown on G.S.M., the rise in pH due to the presence of ammonium ions in solution probably being largely a result of its activity. It is proposed that the oxidation of glutamate is catalyzed in some instances by transamination with oxaloacetate to form alpha-ketoglutarate and aspartate which is then deaminated to fumarate. The activity of this enzyme accounts for the fact that aspartic acid was not detected in the amino acid pool of M. cryophilus grown on G.S.M.

These enzyme assays substantiate the conclusions derived from the earlier tests; no temperature-sensitive lesion has been shown in the area of intermediary metabolism, respiration or oxidative phosphorylation.

The last series of investigations in intermediary metabolism consisted of feeding labelled precursor to cells and studying the incorporation and distribution of label. A comparison was made of the ability of normal and heat-shocked cells to incorporate 2-^{14}C-acetate at 14^{o}C with the aim of relating differences to temperature-inflicted damage to the organisms' metabolism. The results (Table 7) are expressed as distribution of label rather than distribution of incorporated label as there has been sufficient evidence

Table 7: Distribution of Label in Normal and Heat-Shocked Cells

	Control Cells		Heat-Shocked Cells (68% Viable)	
	^{14}C (cpm)	^{14}C (%)	^{14}C (cpm)	^{14}C (%)
Alcohol-water soluble	634,500	82.2	652,600	84.2
Organic acids	205,100	26.6	198,400	25.6
Sugars	23,100	3	19,400	2.5
Basic and neutral amino acids	64,000	8.3	34,900	4.5
Acidic amino acids	341,500	44.3	260,400	33.6
Volatile on acidification	0	0	118,500	18
Residue	77,100	10	62,000	8
CO_2	48,600	6.3	54,200	7
TOTAL ^{14}C	771,000	98.5	775,000	99.2

so far to demonstrate that although cells are non-viable according to the criterion described, they can still carry out many cellular functions apart from reproduction. Consequently, 'dead' cells will take up and utilize the acetate.

The main difference between the two lies in the category, 'volatile on acidification', which may contain small amounts of carbonates or volatile acids, but by far the main component is residual 2-^{14}C-acetate in the medium. Hence the heat-shocked cells in the 20 minute incubation period incorporate only 82% of the added label whereas normal cells incorporate 100%. The percentage incorporation into sugars, organic acids, insoluble fraction and carbon dioxide evolved is comparable in both types of cell. The total amino acid fraction of the control cells contain 52.6% of the added radioactivity, but only 38.1% in the heat-shocked cells. This difference in incorporation accounts for almost all of the percentage label not taken up by the latter cells.

Earlier studies on the effect of moderate temperature on the amino acid pool of ATCC 15174 (Table 5; Figure 18) had shown that amino acids accumulated with time. In the latest experiments, cells recovering from the effects of exposure to moderate temperatures did not seem to channel their flow of metabolites into the synthesis of amino acids, possibly because those which had accumulated were now being utilized. These considerations, plus the observation that

ATP accumulates in cells at 30°C, presumably as it is not being employed in cellular reactions, led to a further area of investigation, macromolecular synthesis and breakdown.

Studies on Macromolecular Synthesis and Breakdown

The effect of moderate temperature on the macromolecular synthesis of protein and nucleic acid was studied, in the first place because of the biochemical indications mentioned, at the end of the previous section, and secondly because of the profound role these molecules are known to play in all expressions of cellular life. Consequently any environmental factors such as temperature, affecting processes like replication, transcription, translation and macromolecular turnover, will cause repercussions throughout the cell.

Initially, the total DNA, RNA and protein contents of cells, incubated at 14°C or 30°C for a short period of time, were estimated. During balanced growth the differential rate of RNA synthesis is determined by the overall growth supporting ability of the medium and is a function of the growth rate. This phenotypic character is extraordinarily susceptible to environmental modification and investigations have shown that, over a wide range of growth rates, the fraction of a cell's resources allocated to RNA synthesis is a function of the growth rate at a given temperature (Schaechter, Maaloe and Kjeldgaard, 1958; Neidhardt and Magasanik, 1960). When growth rate of a culture is manipulated by environmental alterations, the synthesis of total RNA, and specifically ribosomal RNA (rRNA) is readjusted almost immediately, that of protein and DNA following later. Therefore studies on the effect of transfer

of ATCC 15174 from 14°C to 30°C should result in an increase in RNA level, or a cessation of RNA accumulation depending upon whether or not the effect of moderate temperature on this organism affects macromolecular synthesis. The results of such an experiment are recorded in Table 8 which lists the accumulation of macromolecules, as detected by colorimetric assay, in ATCC 15174 growing in G.S.M. at 14°C and 30°C. These measurements had to be related to the loss of cellular viability at moderate temperature, thus samples were withdrawn only for 45 minutes. The growth rate of ATCC 15174 is slow in G.S.M. at these temperatures and over the experimental period only small relative increases were recorded. However, it appeared that macromolecular synthesis was greatly affected on change up to 30°C, the relative concentration of RNA and the RNA:protein ratio both falling with viability. Similar experiments were carried out with the mesophilic mutant grown in TSB to promote quicker growth and hence faster changes in macromolecular concentrations over the 45-minute test. Both at 14°C and 30°C this strain showed accumulation of all 3 macromolecules, indicating adjustment to the conditions.

Measurements of macromolecular synthesis by isotope incorporation confirmed the colorimetric data. (Figures 22 and 23). ATCC 15174 when fed ^{14}C-leucine or ^{14}C-uracil incorporated this material into protein and nucleic acids respectively at a constant rate at 14°C. When ^{14}C-leucine was added at the time of transfer to 30°C however, the rate of incorporation matched that at 14°C for 15 minutes and

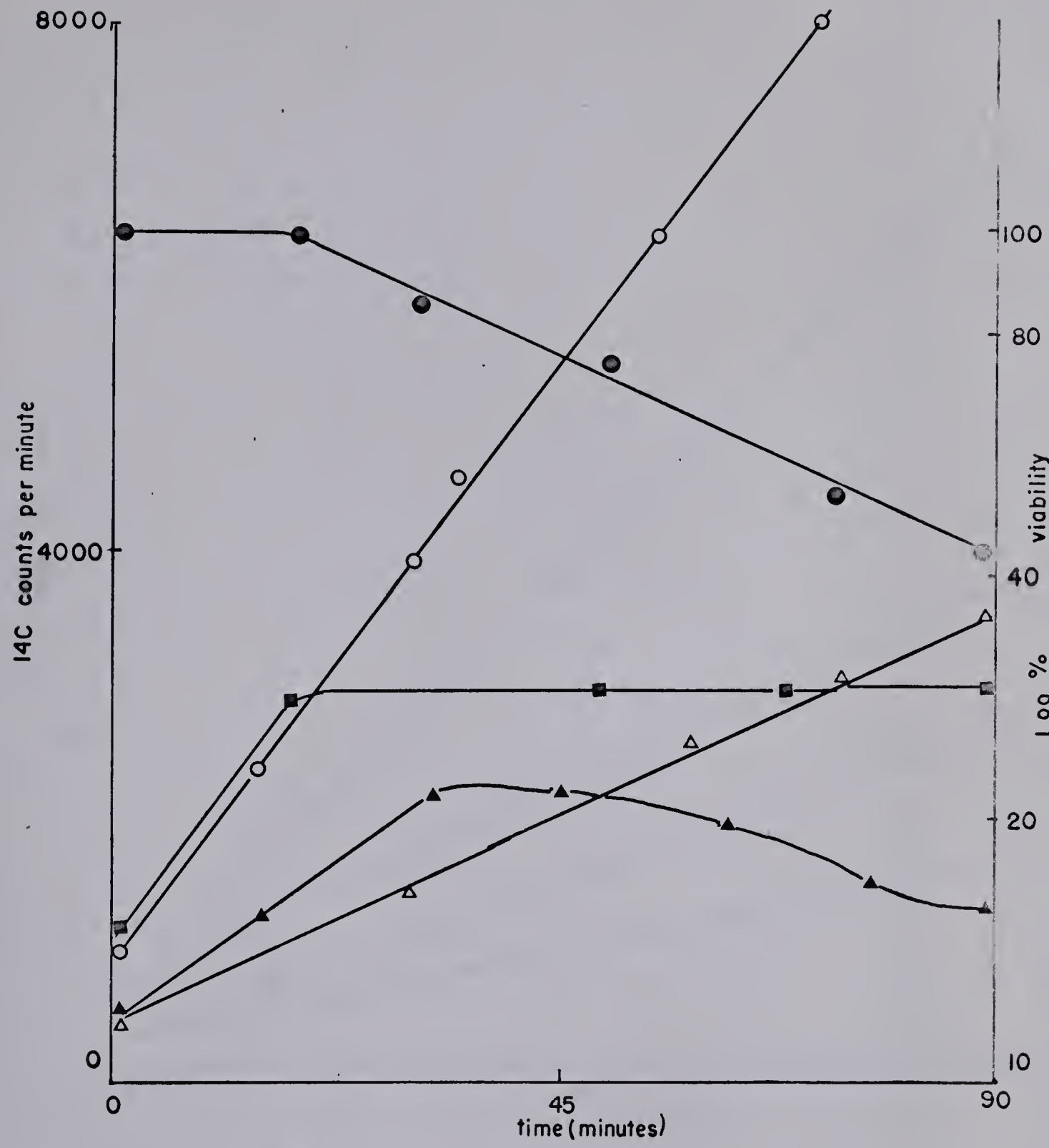
Table 8: Accumulation of Deoxyribonucleic Acid, Ribonucleic Acid and Protein by ATCC 15174 and 15174-RB at 14°C and 30°C

Organism	Temperature of Incubation	Time at Specified Temp.(min.)	DNA*	Protein*	RNA*	RNA: Protein*	Percent Viability
ATCC 15174[X]	14°C	0	1.00	1.00	1.00	1.00	100
		20	1.10	1.14	1.12	0.98	108
		45	1.20	1.31	1.22	0.93	119
ATCC 15174[X]	30°C	0	1.00	1.00	1.00	1.00	100
		20	1.00	1.10	0.94	0.85	89
		45	1.10	1.00	0.82	0.82	76
15174-RB[+]	14°C	0	1.00	1.00	1.00	1.00	100
		20	1.09	1.10	1.14	1.03	105
		45	1.18	1.35	1.27	0.94	109
15174-RB[+]	30°C	0	1.00	1.00	1.00	1.00	100
		20	1.35	1.31	1.37	1.05	112
		45	1.72	1.73	1.82	1.05	132

* All values are normalized to the value at zero time to show relative changes.

X Grown in G.S.M.

+ Grown in T.S.B.



■—■ 14C-Leucine, 30°C.
○—○ 14C-Leucine, 14 °C.
△—△ 14C-Uracil, 14° C.
▲—▲ 14C-Uracil, 30°C
●—● Log % viability, 30°C.

FIG 22: EFFECT OF TEMPERATURE OF INCUBATION ON THE INCORPORATION OF 14C-Leucine or 14C-Uracil INTO MACROMOLECULES OF ATCC 15174; AND RELATIONSHIP TO VIABILITY.

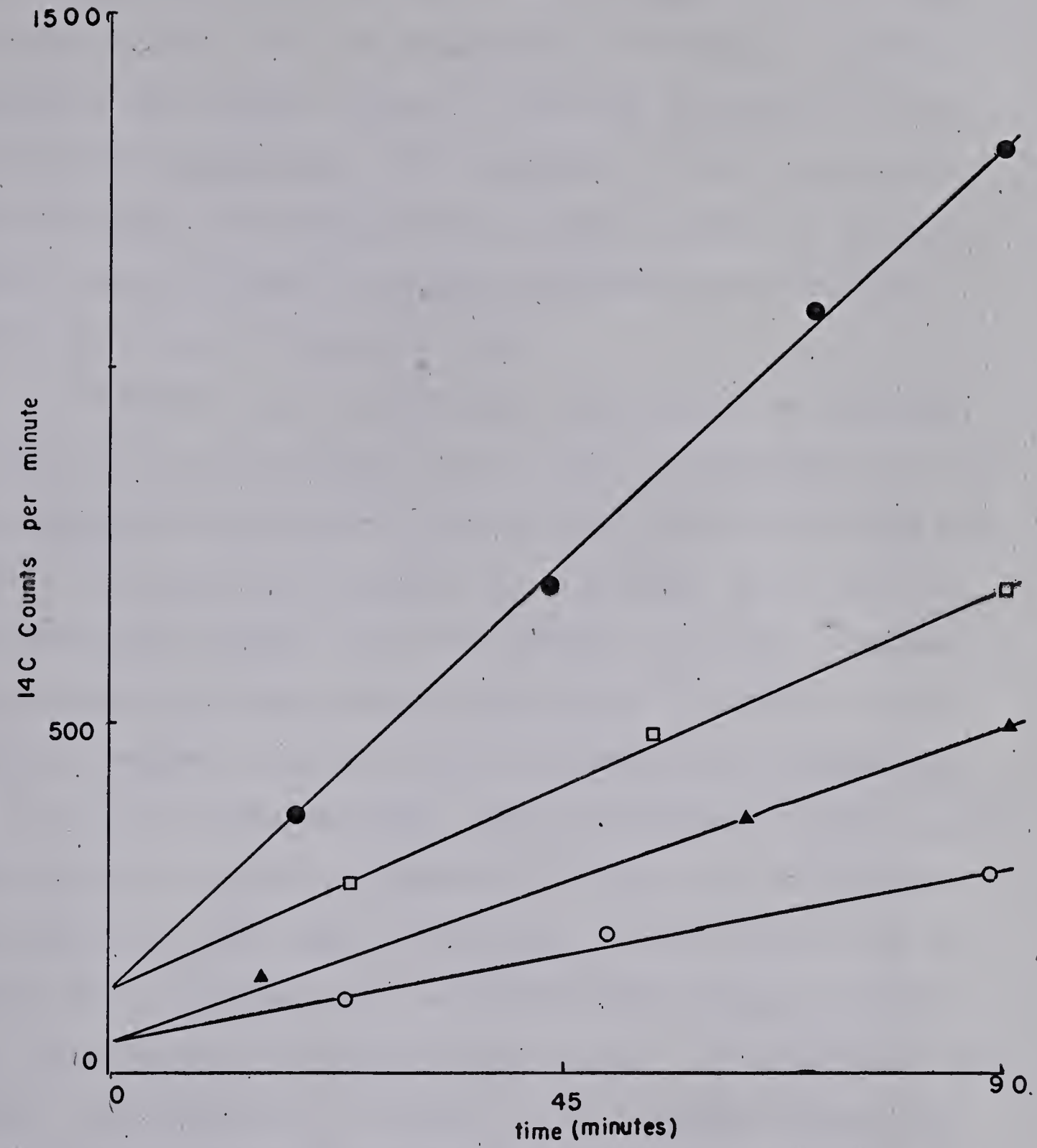


FIG.23: EFFECT OF TEMPERATURE OF INCUBATION ON INCORPORATION OF 14C-Leucine & 14C-Uracil INTO MACROMOLECULES OF 15174-RB.

then halted abruptly and there was no change in the level of incorporation for the remainder of the test. Protein synthesis was halted before it had time to adjust to the new growth temperature. On comparison of this curve with that denoting the viable state of the culture, it is noteworthy that cessation of protein synthesis and loss of viability occur at the same time.

^{14}C-uracil was incorporated immediately on transfer to 30°C at a rate greater than at 14°C, indicating that some adjustment had been made following the change-up. This continued for almost 30 minutes, or 15 minutes after leucine incorporation stopped and then halted, and after a further 15 minutes stationary period, the total ^{14}C-uracil incorporated dropped, probably due to turnover and degradation of some of the labelled RNA. This inhibition of both types of synthesis, and the correlation of the halt in leucine incorporation with loss of viability of the cells, was observed with different cell concentrations giving varying lag times before the death of the cells. By comparison, the mutant 15174-RB which is viable at both temperatures also incorporated both types of label at these temperatures, the increased rate at 30°C reflecting the increased growth rate of the cell.

The inability of the psychrophile to continue incorporation of isotopes into macromolecules was demonstrated not to be due to an inability to transport the amino acid or the base across the membrane (Figure 24). Total uptake

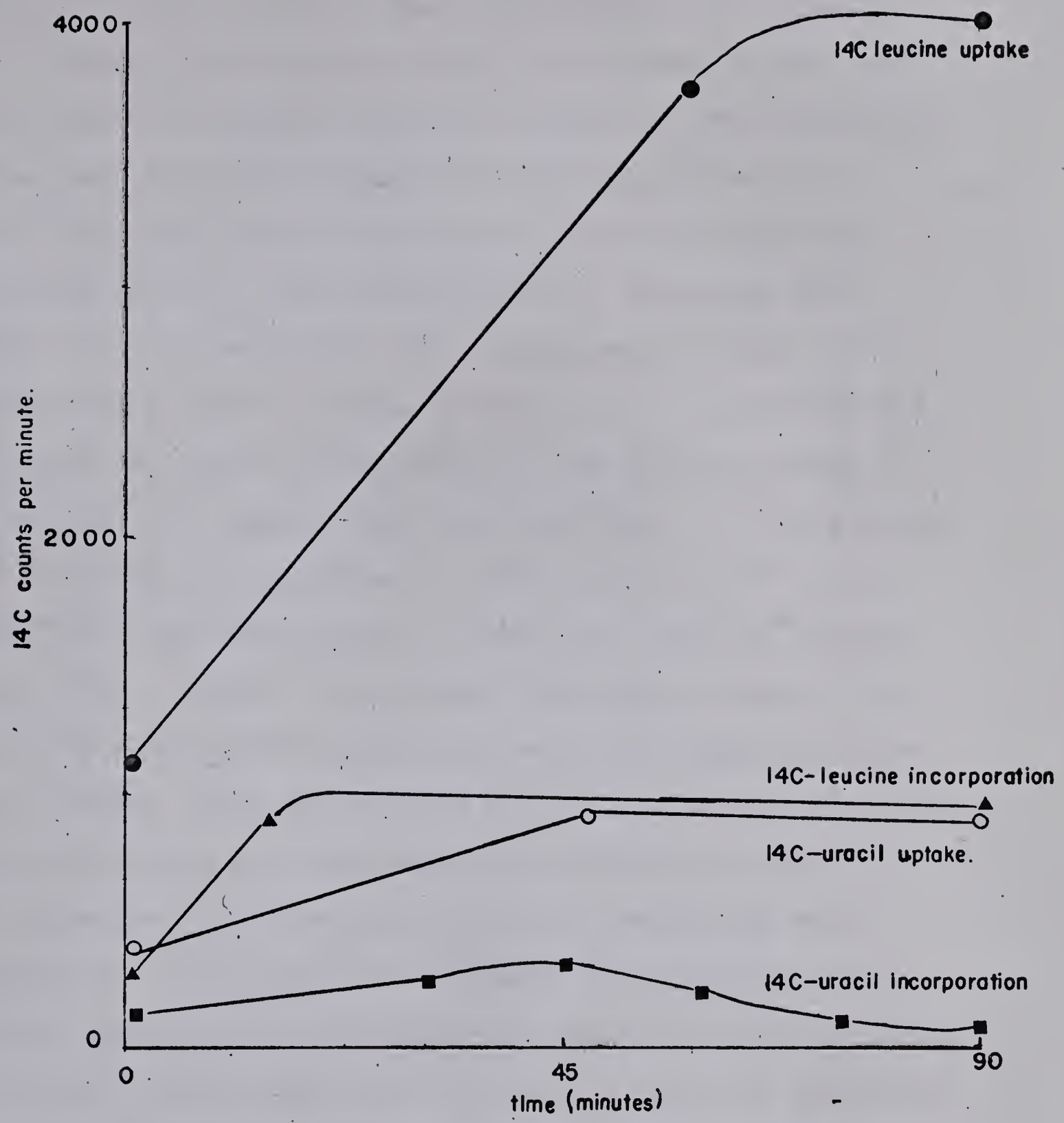
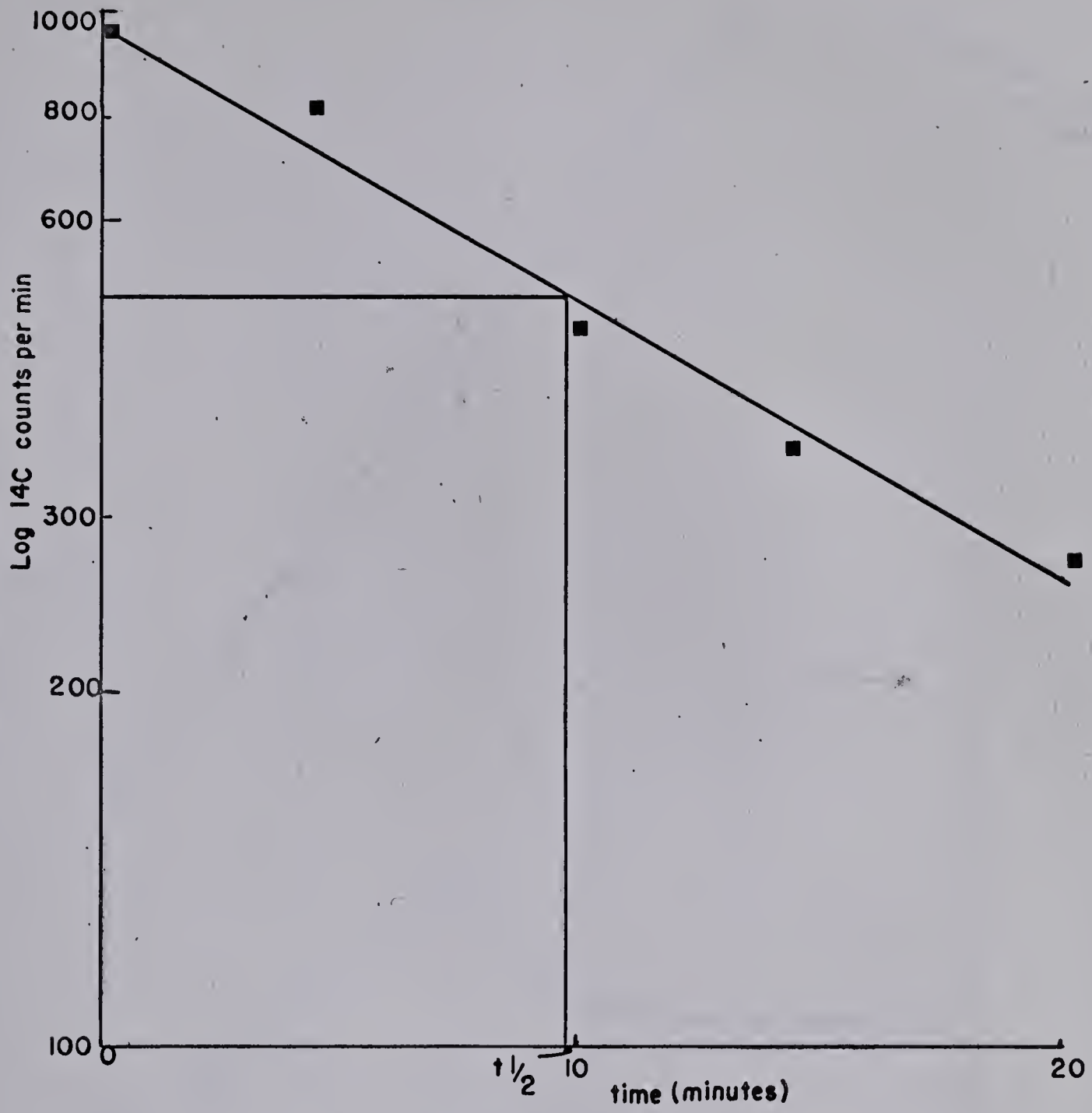


FIG.24: EFFECT OF MODERATE TEMPERATURE ON UPTAKE AND INCORPORATION OF 14C-leucine AND 14C-uracil BY ATCC 15174.

continues after the cell loses its reproductive capacity.

Midgely and McCarthy (1962), in studies on the metabolic role of a rapidly-labelled fraction of RNA appearing in the cell following pulse labelling with ^{14}C-uracil, noted that this fraction was heterogenous and could be separated into 30 - 40% DNA-like RNA or messenger RNA (mRNA), and the rest (60 - 70%) ribosomal-like RNA. This mRNA fraction had an average lifetime of 2 - 3 minutes and then lost its label by degradation, the products being incorporated into soluble RNA (sRNA) and DNA. In the studies on ^{14}C-uracil incorporation at 30°C in ATCC 15174 it was noted that there was uptake of label for abour 30 minutes then, after a further 15 minutes, there was a loss of isotope from the cold TCA-insoluble material, suggesting turnover of mRNA. This was studied at 14°C, first by the kinetic method measuring the time required to remove an amount of RNA equivalent to the size of the pool inside the cell (Figure 25). The rate of disappearance of radioactivity from pre-labelled RNA, to which non-radioactive precursor has been added, is depicted and $t_{1/2}$ the half-life of the decay process is measured. From this, t_t or turnover time has been calculated to be 15-16 minutes. Use of the time interval method (Figure 26) showed that the incorporation of leucine in protein ceased approximately 15 minutes later than the incorporation of uracil into RNA when synthesis of RNA was inhibited by actinomycin D. It appears then that after inhibition of protein and RNA synthesis at 30°C, mRNA is



$t_{1/2}$ = 9·75 minutes

$t_t = t_{1/2} \times \frac{1}{\ln 2}$

= 15 minutes.

FIG. 25: TURNOVER OF PRELABELLED MATERIAL IN R.N.A. OF ATCC 15174 AS MEASURED BY INCORPORATION OF NON-RADIOACTIVE PRECURSOR.

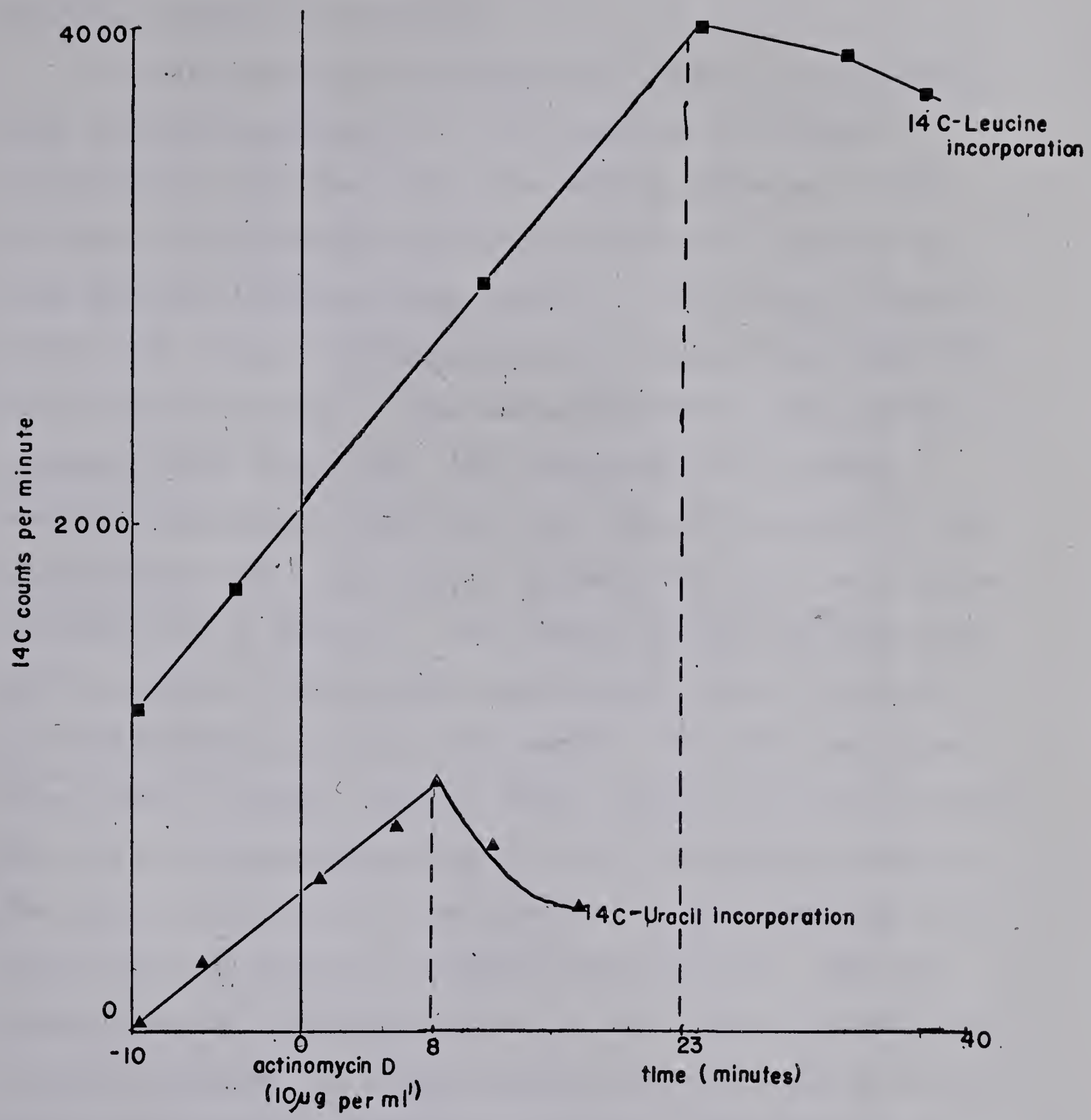


FIG.26: TIME INTERVAL BETWEEN INHIBITION OF 14C-uracil INCORPORATION & INHIBITION OF 14C-leucine INCORPORATION IN THE PRESENCE OF ACTINOMYCIN D.

degraded without re-synthesis.

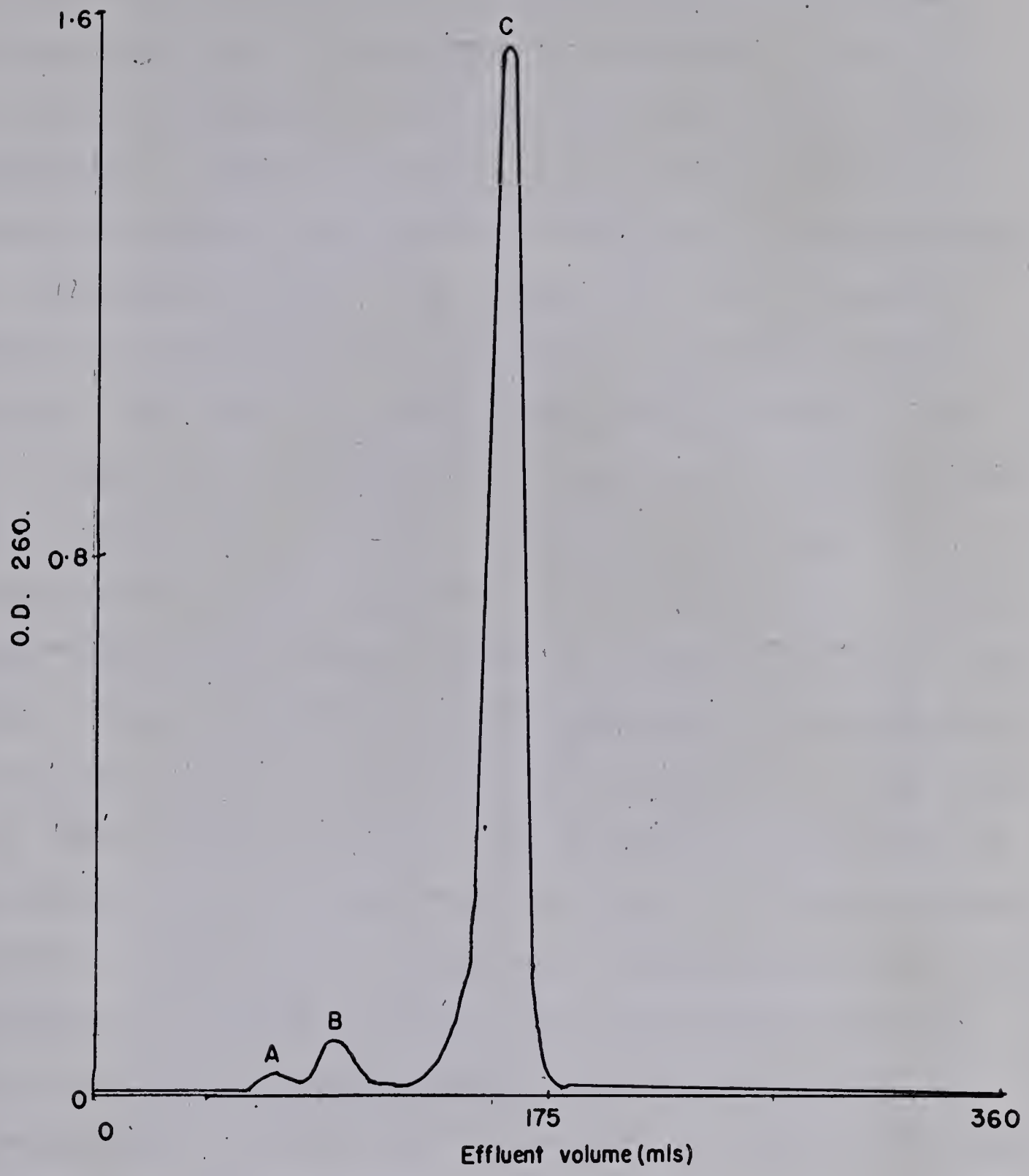
Earlier studies (see Figure 14, Table 4) established that extended incubation at 30°C resulted in leakage of intracellular materials into the medium, principally 260 millimicrons-absorbing and orcinol-positive. These materials were now examined more closely. A four-hour incubation period was chosen, chemical analyses showing that there was little contamination of the medium with DNA, amino acids, or protein within this time. The distribution of orcinol-reacting materials between hot and cold TCA extracts of the supernatants and a cell lysate prepared by sonic oscillation is described in Table 9. The control culture maintained at 14°C in buffer released very small quantities of cold TCA-soluble material only into the medium. The 30°C cell-free supernatant, however, shows a small amount of hot TCA-soluble RNA and a considerable amount of cold TCA-soluble material. The ratio of the cold to hot fraction is 18:1 implying that there has been selective leakage from the cell. This was established by comparing this ratio with that obtained using a lysate prepared for normal cells; here the ratio is 1:0.63. Thus considerable breakdown of polymeric RNA must have occurred during the 4-hour incubation period, with little autolysis.

This material in the cell-free supernatant was examined further by fractionation on Sephadex G-25 (Figure 27). Three peaks are detectable: an excluded volume (A), corresponding to a peak produced by highly polymerized yeast RNA

Table 9: Distribution of Orcinol-Reacting Materials in Hot and Cold TCA Extracts of Cell Lysate and Cell-Free Supernatants

Preparation	Cold TCA Extract micrograms RNA*	Hot TCA Extract micrograms RNA*	Ratio (Cold Extract/ Hot Extract)
14°C Cell-Free Supernatant	5	0	-
30°C Cell-Free Supernatant	48	2.5	19:1
Cell Lysate	228	144	1:0.63

*Results for supernatants are expressed per ml. of supernatant; for cell lysate, per ml. of a 1:30 dilution of lysate.

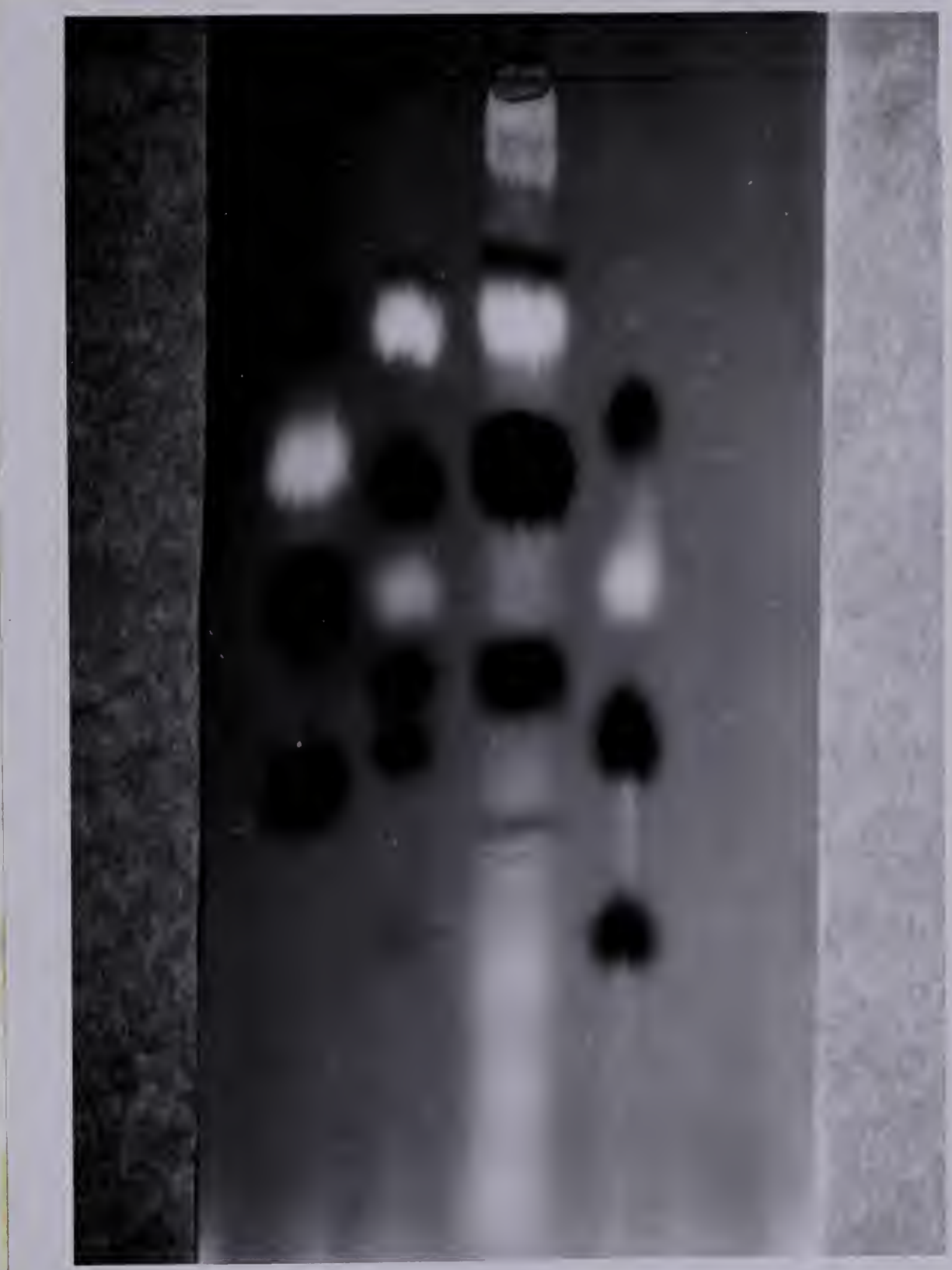


Peak A— Totally excluded peak corresponding to polymeric R.N.A.

FIG. 27: SEPHADEX G-25 ELUTION PATTERN OF 30° C CELL-FREE SUPERNATANT.

on a control fractionation; a second peak (B) which was unidentified; and a third peak (C) comprising almost all of the 260 millimicrons-absorbing material present in the supernatant. Selective leakage was indeed occurring, one species of RNA as determined by molecular size accumulating in the medium at 30°C. The volume under peak C was desalted and examined more closely by qualitative chromotography. The resulting chromatogram was illuminated with U.V. light (253 millimicrons) to help detect the separated spots (Figure 28). At this wavelength, guanosine derivatives reflect light, appearing white, while those of adenosine, uracil and cytosine absorb it, appearing black on the sheet. Three mixtures of marker compounds corresponding to 3'-nucleotides, 5'-nucleotides and nucleosides of the four, main bases found in RNA were run alongside the unknown for reference. It is obvious that the sample is a heterogeneous mixture of the four 5'-nucleotides, indicating considerable degradation of polymeric RNA. The nature of the material also suggests an active phosphodiesterase, catalyzing by exonucleolytic activity and the release of mononucleotides terminating in 5'-phosphate.

A comparison was made of the total ribonuclease (RNase) activity of cell extracts at 14°C or 30°C to determine whether activity was greatly affected by these temperatures (Table 10).



Photographed Under U.V. Light (253 millimicrons).

Figure 28: One-dimensional Chromatography of Marker Compounds Along with Unknown 260-millimicron-absorbing Material Released From Cell at 30°C.

Table 10: Ribonuclease Activity of Cell Extract of ATCC 15174 at 14°C and 30°C

Temperature of Assay	Specific Activity (micrograms cold TCA-soluble material formed/hour/mg. protein in cell extract)
14°C	9.0
30°C	16.6

The RNases present show almost twice the activity at 30°C that they do at 14°C. This clearly must have, if not a primary effect on loss of cell viability at least a secondary effect, degrading functional RNA.

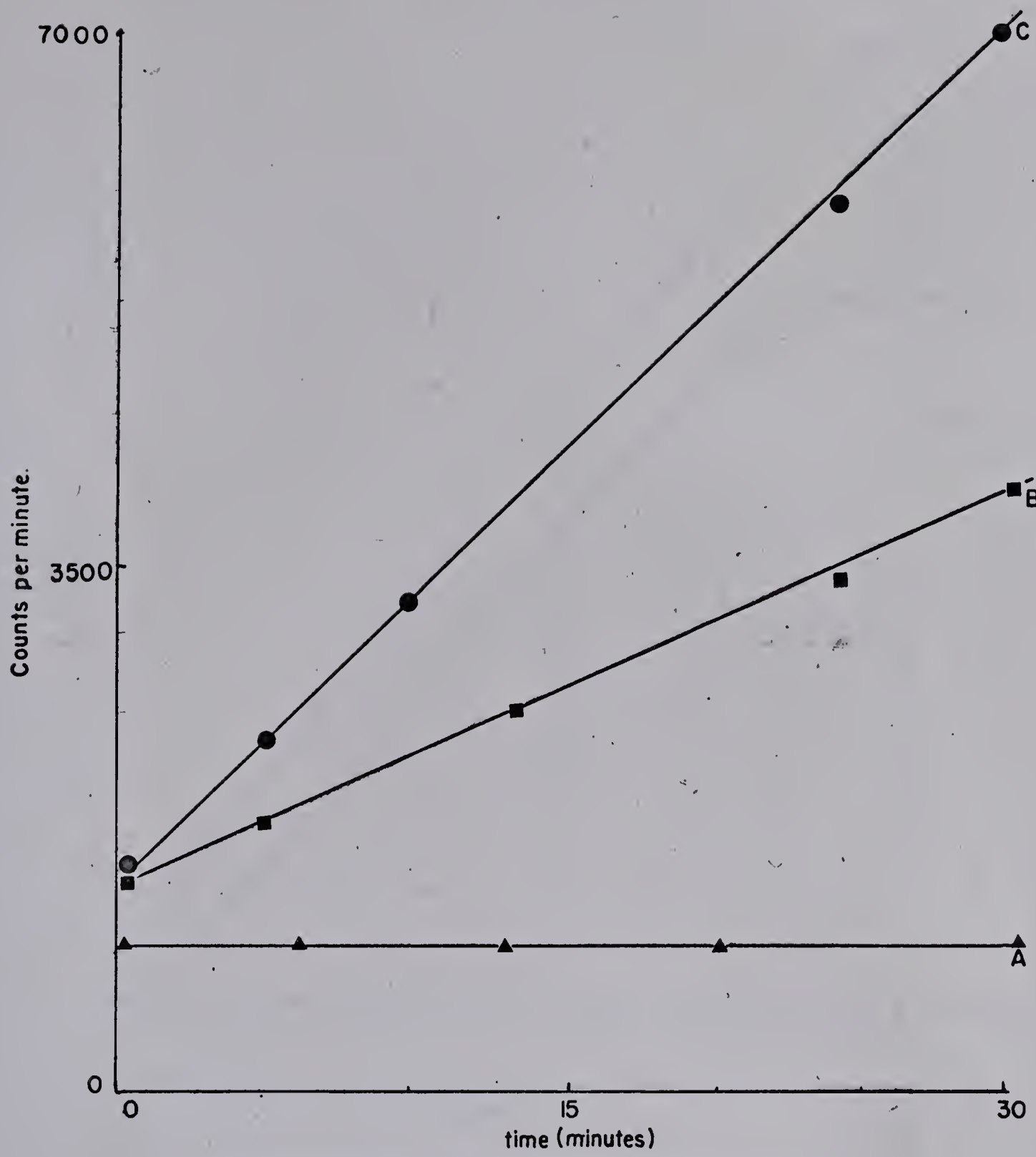
An attempt was made to separate the two phenomena of cessation of protein synthesis and cessation of RNA synthesis at 30°C, to find out the relationship of one to the other. Activation of amino acids and their attachment to tRNA are believed to be obligatory steps in protein synthesis (Simpson, 1962). Amino acids have also been implicated in the regulation of the overall rate of RNA synthesis in bacteria (Neidhardt, 1964), the regulatory function having been visualized by two groups of investigators (Stent and Brenner, 1961; Kurland and Maaloe, 1962) as the neutralization by amino acids of an inhibitory effect of uncharged tRNA on RNA synthesis. The relative efficiency of these amino acid-tRNA-synthetases in crude extracts of ATCC 15174,

were assayed at 20°C and 30°C by the hydroxamate method (Table II). The synthetases for 18 different amino acids were examined, 12 showing little change in activity at the two temperatures, but 6 exhibiting distinct reduction of activity at 30°C. The synthetase activities associated with tryptophan and phenylalanine both fell by about 60%, those of histidine, glutamic acid and aspartic acid by 25%, while alanine-t-RNA synthetase activity dropped by approximately 80% on incubation at 30°C.

A second line of investigation into the relationship of protein synthesis to RNA synthesis in ATCC 15174 at moderate temperature made use of the knowledge of the specific mode of action of antimicrobial drugs. Fraenkel and Neidhardt (1961) and Kurland and Maaloe (1962) noted that a wide range of concentrations of chloramphenicol, an inhibitor of protein synthesis, stimulated RNA synthesis in Aerobacter aerogenes, Salmonella typhimurium while protein synthesis halted. The addition of this drug to cultures of ATCC 15174 at 14°C produced a similar effect (Figure 29); protein synthesis halted immediately while RNA synthesis showed a marked increase in rate over a control incubated without chloramphenicol. At 30°C leucine incorporation is again stopped right away (Figure 30). RNA synthesis at this temperature has been shown to be stimulated for the first 15 to 30 minutes by comparison with 14°C synthesis (see Figure 22) and consequently the effect of chloramphenicol is not so marked, but ^{14}C-uracil incorporation

Table 11: Amino Acid-tRNA Synthetase Activities of Cell Extracts of ATCC 15174

Amino Acid	Specific Activity (micromoles hydroxamate formed/hour/mg. protein)	
	14°C Assay	30°C Assay
Alanine	0.011	0.002
Arginine	0.021	0.019
Aspartic Acid	0.038	0.027
Cysteine	0.01	0.01
Cystine	0.014	0.015
Glutamic Acid	0.04	0.03
Glycine	0.011	0.011
Histidine	0.021	0.015
Isoleucine	0.013	0.013
Leucine	0.009	0.009
Methionine	0.011	0.012
Phenylalanine	0.016	0.006
Proline	0.015	0.015
Serine	0.03	0.03
Threonine	0.016	0.015
Tryptophan	0.014	0.006
Tyrosine	0.008	0.008
Valine	0.012	0.013



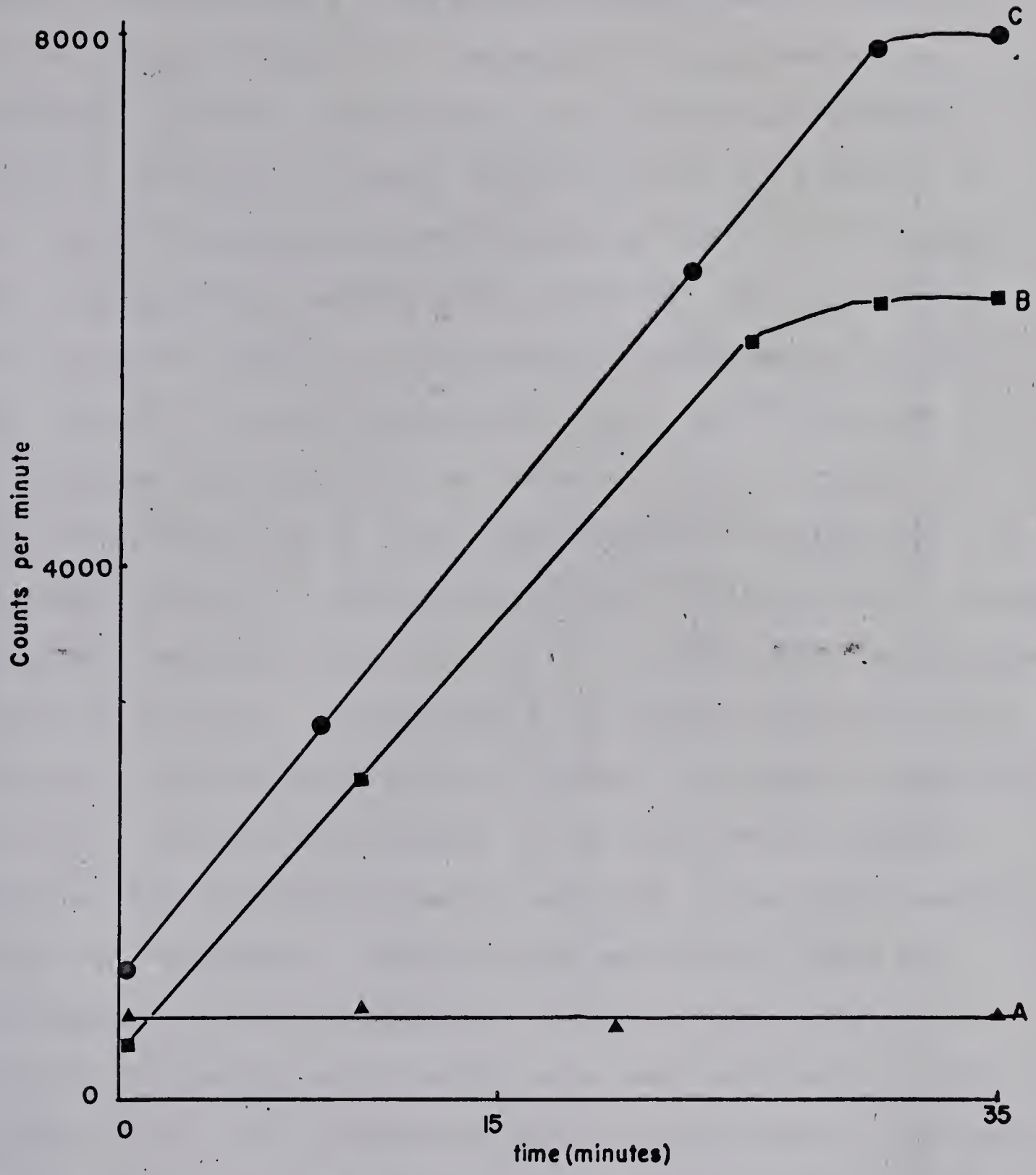
Chloramphenicol — 60 ug per ml final concentration.

A-14C-leucine incorporation; + chloramphenicol

B-14C-uracil incorporation; no chloramphenicol

C-14C-uracil incorporation; + chlordmphenicol

FIG.29: EFFECT OF CHLORAMPHENICOL ON 14C-Leucine & 14C-Uracil INCORPORATION AT 14°C IN G.S.M.



Chloramphenicol – 60µg per ml final concentration.

A 14C–leucine incorporation ; + chloramphenicol.

B 14C–uracil incorporation ; no chloramphenicol.

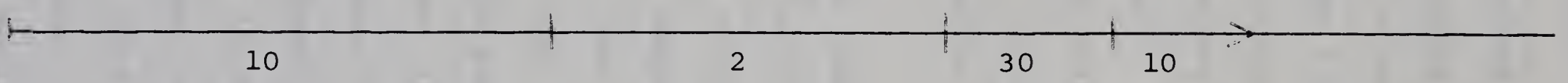
C 14C–uracil incorporation ; +chloramphenicol .

FIG. 30: EFFECT OF CHLORAMPHENICOL ON 14C-Leucine & 14C-Uracil INCORPORATION AT 30°C IN G.S.M.

still continues for a further few minutes before halting. If the effect of moderate temperature on macromolecular synthesis were the inactivation of a particular enzymic step, or sequence of steps, then it should be possible to halt protein synthesis specifically by the use of chloramphenicol and then replace this inhibitor, and its effect, with another namely, an environmental temperature of 30°C. The results of such a sequence of tests on ^{14}C-leucine incorporation are described in Table 12. Control cells at 14°C or 30°C in G.S.M. plus added labelled leucine will incorporate enough of this into the cold TCA-insoluble fraction to give a measured radioactivity of 16,000 counts per minute after 10 minutes. Incorporation of leucine into this fraction was found to halt after a further 5 minutes incubation at 30°C. Where pre-treatment of the cells with chloramphenicol had occurred prior to exposure to the test temperatures for 30 minutes, considerable amounts of label were incorporated in the subsequent 10-minute labelling period. Protein synthesis, which might have been expected to stop midway in the 30°C incubation period, continued at a greatly increased rate for at least a further 25 minutes at 30°C. Clearly, the inhibition of protein synthesis at 30°C is not an isolated phenomenon but is related to other factors, especially RNA synthesis. A fuller discussion of the results presented in this last section and their possible implications will be reserved for the last section of this thesis.

Table 12: Effect of Pre-treatment With Chloramphenicol on Subsequent Incorporation of ^{14}C-Leucine

				Radioactivity Incorporated into cold TCA-soluble fraction (counts per minute)
A). Chloramphenicol Treatment				
+Chloramphenicol (50 micrograms/ml.); 14°C incubation; G.S.M.	Removal of chloramphenicol; cells washed with 14°C G.S.M.	Incubation at 14°C in G.S.M.	+^{14}C-Leucine	37,000
+Chloramphenicol (50 micrograms/ml.); 14°C incubation; G.S.M.	Removal of chloramphenicol; cells washed with 30°C G.S.M.	Incubation at 30°C in G.S.M.	+^{14}C-Leucine	81,000



(Time scale in minutes)

B). Control

Incubation at 14°C in G.S.M. + ^{14}C-Leucine for 10 minutes	16,000
Incubation at 30°C in G.S.M. + ^{14}C-Leucine for 10 minutes	16,000

Extended incubation of the 30°C control showed that incorporation of Leucine halted after 15 minutes.

CONCLUSIONS AND DISCUSSION

This investigation has been concerned primarily with one aspect of the physiology of Micrococcus cryophilus, its inability to grow above 25°C. The biochemical reason or reasons for this phenomenon have been studied, emphasis being placed on the changes occurring during the onset of lethality rather than examining all factors pertaining to loss of viability at moderate temperature. The standpoint taken at the beginning of the study was that there must be a primary, temperature-induced, lesion halting growth, after which secondary factors may influence the death rate of the cell.

The organism has been shown to be capable of growth on a basic, minimal medium containing L-glutamic acid as sole nutrient source, and incapable of utilizing glucose and other carbohydrates. This minimal medium supports the growth of ATCC 15174 up to 25°C but no form of supplementation was found to sustain growth above that temperature. These results indicated that temperature-sensitivity of the organism was probably not due to the blocking of a specific biosynthetic pathway or sequence of pathways. Hagen and Rose (1962) have reported a psychrophilic cryptococcus which could only grow at 30°C if cellular reserves of keto acids were supplemented at higher temperatures.

A mesophilic mutant was isolated from ATCC 15174, capable of growth to a maximum temperature of 34°C. This strain grew more slowly in the minimal medium than the

psychrophile, but very well on complex medium. If the mesophilic character had been due to an ability to synthesize, in limiting amounts at 30°C, a metabolite unavailable to the psychrophile because of a specifically heat-sensitive synthesizing system, then it might have been possible to pinpoint the area affected by supplementing the minimal medium with specified metabolites and noting which improved growth the most significantly. No such specific effect was found.

Another explanation postulated for the low maximum growth temperatures of obligate psychrophiles is heat-induced damage of the cellular membrane leading to leakage of intracellular material (Morita and Burton, 1963; Haight and Morita, 1966). These workers noted that on heat-shocking a psychrophile in buffer, protein, DNA, RNA and amino acids were leaked into the medium. They did not, however, establish the exact relationship between leakage and loss of viability as did Hagen et al. (1964) who, studying a different psychrophile, indicated that loss of viability preceded lysis. ATCC 15174 in buffer at 30°C does release material into the medium, first RNA and a little protein, but only after approximately 90% of the culture is non-viable do large amounts of RNA, protein and DNA become detectable, this increasing release occurring about the same time as cell lysis, as measured by optical density. Only at this stage, too, does the cell become permeable to a protein-conjugating, fluorescent dye.

Electron microscope studies revealed no gross damage to the surface of the cells as they became non-viable at 30°C. Leakage of functional macromolecules from the cell is not a cause but rather a consequence of death. An analysis of the RNA released over the first four hours of incubation at 30°C revealed that it had been extremely degraded within the cell and virtually only 5'-nucleotides of the bases adenine, guanosine, cytosine and uracil were detected in the medium. This implies some form of selective leakage, indicating that membrane integrity is not yet affected by the moderate temperature.

Temperature-sensitive enzymes of the TCA cycles of several psychrophilic bacteria have been reported (Evison and Rose, 1965; Upadhyay and Stokes, 1963; Burton and Morita, 1963) although these observations were not related specifically to the determination of the maximum temperature for growth in each of these organisms. Several pieces of evidence helped establish the fact that energy metabolism in ATCC 15174 is not restricted by the presence of any exceedingly heat-sensitive enzymes. Studies on the amino acid and keto acid pools of these organisms at 30°C showed an initial fall in level in the former for a short time then a gradual rise in level, despite the continued depletion of glutamic acid, due to accumulation of other amino acids; and a continuing fall in the level of the latter. These findings can be partly explained by the results of respiratory and coupled phosphorylation studies.

Total oxygen uptake in the presence of an oxidizable substrate was not affected by temperature but, at 30°C, endogenous respiration comprised a greater proportion of the total. ATP synthesis continued in the presence of added glutamate and eventually began to accumulate very quickly after 4 to 5 hours. These tests were complemented by assays on the activities of the TCA cycle enzymes in this organism, as well as those pertaining to glutamate metabolism. No evidence of any temperature-sensitivity was found, either in cell extracts assayed at 30°C or in extracts prepared from cells which had been exposed to 30°C. In fact, several enzymes exhibited greater activity at 30°C than at 14°C.

Incorporation studies comparing normal and heat-shocked cells showed that the latter only incorporated 80% of the label incorporated by the former. The distribution of the label within the cellular fraction showed one further difference, a significantly smaller proportion had been incorporated into the amino acids, basic, neutral or acidic. This might be construed as reflecting the accumulation of amino acids during prior exposure to 30°C. If neither amino acids nor ATP are utilized at 30°C, then protein synthesis may be a factor.

Macromolecular synthesis was studied by colorimetric analyses and by following the incorporation of labelled leucine into the cold TCA-insoluble fraction of cells. At 30°C first protein synthesis then RNA synthesis halted, and

breakdown of the rapidly labelled fraction of RNA ensued. The relevance of these findings to the question of cellular viability is obvious when they are compared with the results of viable counts performed on samples withdrawn at regular intervals throughout the test; loss of viability within the culture commences as protein synthesis halts. As has been stated, the criterion of viability in these studies has been ability of the cells to reproduce. This implies DNA replication, for which protein formation (Pardee and Prestidge, 1956; Gros and Gros, 1956; Maaloe and Hanawalt, 1961) and RNA synthesis (Doudney, 1966) have been cited as essential requirements. Hence the significance of this observation to the question of temperature-induced death.

Further studies on the subsequent breakdown of the rapidly-labelled RNA fraction revealed that the loss of radioactivity from this material commenced within 15 minutes of inhibition of synthesis. Such a fraction has been described as comprising approximately 40% DNA-like RNA or mRNA and approximately 60% precursors of mRNA (Midgely and McCarthy, 1962). If it is assumed that the tRNA is stable under these experimental conditions, then relatively rapid degradation of the labelled polymeric material might be attributed to the turnover of mRNA. This alone would ensure that no further protein synthesis would occur had it not halted already. The determination of turnover of labile RNA by the method of Tarver (1954) was based on three

assumptions: a) that the cellular material investigated is a homogenous mixture from which the radioactive components are lost in a random manner; b) on release of the isotopic components into the intracellular pool, it is believed that they are diluted by non-radioactive components and that the reincorporation of radioactivity is negligible. Obviously these two assumptions are not valid. Messenger RNA forms a heterogeneous mixture, and the life span of the components will probably differ; therefore the life span value obtained will depend upon the experimental conditions and the metabolic state of the cell. As it is believed that the degradation merely emphasises the fact that protein synthesis is halted, these objections become less important; c) it was assumed that uracil is incorporated into RNA only. This is not absolutely correct. It has been shown (Richardson, Schildkraut and Kornberg, 1963) that uracil can be incorporated into DNA. If it is assumed that the turnover of DNA is much slower than that of RNA, then this fact becomes less important.

The time-interval method was also used for the measurement of the life span of labile RNA, using actinomycin D to inhibit RNA synthesis. The validity of this technique has been established by Chantrenne (1965). Using these techniques a turnover time of approximately 15 minutes was obtained for mRNA in the psychrophile growing normally at 14°C. A somewhat similar life span for the biological activity of this macromolecule might be expected at 30°C, despite the

lack of protein synthesis. Edlin and Maaloe (1966) have shown, in studies on the synthesis and breakdown of alkaline phosphatase mRNA, that the rates of synthesis and breakdown of this message are almost the same with or without protein synthesis, suggesting that transcription and translation are not necessarily coupled. Only under conditions of energy starvation, which do not apply here, did Fan, Higa and Levinthal (1964) detect any protection of the mRNA of Bacillus subtilis from normal decay.

Protein turnover in the cells was not determined as it was obvious that many catalytic functions were carried out throughout the cell at 30°C, despite the fact that viability had been lost. The inhibition of protein synthesis at this temperature would only prevent the cell synthesizing the new protein required for replication of DNA and reproduction. Mandelstam and Halvorson (1960), examining the stability of protein in non-growing E. coli, found that there was balanced degradation and re-synthesis at the rate of about 5% per hour at 35°C. ATCC 15174 at 30°C might even show a lower rate of degradation although the possibility that the activity of intracellular proteolytic enzymes is increased at this temperature, must not be discounted.

During the earlier part of the work it had been noted that ribonucleic acid-like material was leaked from cells of ATCC 15174 in measurable quantities at 30°C, in buffer. This occurred several hours before other intracellular

components appeared in the supernatant, therefore autolysis was discounted. An investigation into the nature of this material yielded several very interesting results. Virtually all of it was soluble in cold TCA and, following molecular sieving on Sephadex, this fraction was collected under one major peak. Chromatography showed this to be a heterogeneous mixture of 5'-nucleotides of the four main bases in RNA. No thymidine-5'-phosphate was detected. These results suggested the presence of a phosphodiesterase, active against RNA which is degraded non-specifically by exonucleolytic action from the 3'-hydroxyl terminal, releasing sequentially the individual 5'-nucleotides. Such an activity would be somewhat comparable to that described by Razzell and Khorana (1959 a and b) for snake venon phosphodiesterase which is active against ribonucleotide and deoxyribonucleotide polymers. The release of these nucleotides from cells incubated at 30°C might be the result of a phosphodiesterase specifically activated at the higher temperature. Indeed, the RNase activity of cell extracts, as measured by the increase in cold TCA-soluble material, was shown to be greater at 30°C than at 14°C but this of course is a measure of the total activity of all enzymes capable of degrading RNA and not just this exonuclease. The possible significance of this phosphodiesterase in the temperature-sensitive response of the cell will be discussed below.

The relationship between the inhibition of protein

synthesis and the inhibition of RNA synthesis in ATCC 15174 at 30°C was investigated initially by studying the activation of amino acids in the presence of ATP, magnesium ions and specific enzymes, with the formation of enzyme-bound amino acyl adenylates, the first intermediates in the process of protein synthesis. The amino acyl group of the enzyme-bound complex is transferred to an amino acid-specific tRNA where it is attached by an ester linkage to the ribose portion of the terminal adenosine residue (Moldave, 1965). This sequence of events is catalyzed by an amino acid-tRNA synthetase which plays a role not only in protein synthesis, but also in the regulation of RNA synthesis. Stent and Brenner (1961) and Kurland and Maaloe (1962) have proposed that amino acid-free tRNA is a specific repressor of RNA synthesis, and that amino acyl tRNA is inactive as a repressor. Hence, any factor disturbing the binding of the amino acid to tRNA would not only prevent protein synthesis, but also eventually affect RNA synthesis. An assay of 18 of the amino acid-tRNA synthetases at 20°C and 30°C revealed that at the higher temperature six of these enzymes - those specific for tryptophan, phenylalanine, histidine, glutamic acid, aspartic acid and alanine - had reduced specific activities. These activities were estimated by the chemical reaction between hydroxylamine and the activated amino acid. Whether they represent activities reduced generally at the higher assay temperature, or whether complete inactivation occurred following

a short period of normal activity is not known. However, for the reasons stated, this is a significant finding although the bearing of other factors has yet to be considered.

There is some evidence to support such an effect on protein and nucleic acid syntheses by temperature-inactivated amino acyl-tRNA synthetases. Neidhardt, as part of a broad programme of study on nucleic acid synthesis and its regulation has isolated temperature-sensitive mutants of E. coli with altered polymerases or malfunctioning regulatory devices. Early reports on this work (Fangman and Neidhardt, 1964; Eidlic and Neidhardt, 1965; Bock, Faiman and Neidhardt, 1966) have described mutants with an amino acyl-tRNA synthetase functioning normally at 30°C, but not at 37°C.

A final approach to the question of the relationship between protein and nucleic acid syntheses, and the effect of moderate temperature on these operations, was made by employing the drug chloramphenicol.

Kurland and Maaloe (1962) observed that when cells of E. coli were grown in minimal medium, the addition of chloramphenicol produced an apparently instantaneous doubling of the rate of synthesis of all three classes of RNA. A state of unbalanced growth had been created in which RNA synthesis was kinetically dissociated from protein synthesis due, perhaps, to an increase in intracellular amino acid concentration. Their hypothesis concerning general

RNA synthesis regulated by the availability of amino acids to act as inducers by combination with the appropriate tRNA's, and the evidence for this, need not be discussed at this time (see Maaloe and Kjeldgaard, 1966 for a thorough discussion of current knowledge of RNA regulation). It has been shown that for many kinds of cells, the faster they grow, the greater is their total RNA content (Brachet, 1955). The RNA:protein ratio of bacterial protoplasm is a direct function of the growth rate, within a certain range, at a given temperature (Schaechter, et al. 1958; Neidhardt and Magasanik, 1960). Thus, the pattern according to which cellular metabolites are apportioned for the synthesis of RNA, DNA or protein appears to be sensitive to the environment, rather than rigidly determined. A change in the rate of RNA synthesis results in a change in the rate of protein and DNA synthesis a few minutes later.

When chloramphenicol was added to a culture of ATCC 15174 at 30°C, protein synthesis was inhibited immediately while RNA synthesis was stimulated for a short time, both at 14°C and 30°C, although this effect was not continued at the higher temperature. If cells were briefly exposed to chloramphenicol at 14°C and then removed from the antibiotic, subsequent incorporation of ^{14}C-leucine at 14°C was considerably enhanced due to the presence of the accumulated RNA. If incubation following chloramphenicol removal was at 30°C, then even more RNA accumulated due, not only to the antibiotic effect noted, but also to the increased rate

of synthesis for the first halfhour at the higher temperature. Instead of ^{14}C-leucine incorporation halting after 15 minutes at 30°C as happened with an untreated control, incorporation contined, at a high rate, for at least a further 25 minutes. Protein synthesis at 30°C appears to be related, as at 14°C, to the amount of RNA present in the cell and although it is halted first following transfer this, in actuality, may be a response to temperature-induced modification of some aspect of RNA synthesis or function.

The investigation into the biochemical basis of extreme temperature-sensitivity of M. cryophilus concludes, then, that this is due to a lesion pertaining to macromolecular synthesis in the cell. The primary effect of moderate temperature would appear to be in some aspect of RNA synthesis or regulations, with repercussions, quickly detectable, in protein synthesis.

More detailed conclusions cannot be drawn at this stage, but possible molecular explanations will be discussed, in the spirit of working hypotheses for further investigation. Several of the references cited have pointed to the importance of the amino acid-tRNA synthetases and tRNA's in the regulation of certain cellular activities, in particular the dual role played by the reaction between these molecules in both protein synthesis and in regulation of RNA synthesis. The finding of several amino acid-tRNA synthetases with reduced activities at 30°C is important in the light of the work of Neidhardt and his colleagues. Much has yet to be learned about the steps preceding peptide polymerization:

the activation of the amino acid, its esterification to tRNA, and the interaction of the anti-codon site of tRNA with the codon of the mRNA on the ribosome. In this tripartite sequence, the tRNA plays a key role, recognizing the appropriate aminoacyl synthetase by a mechanism at present unknown, and subsequently positioning the esterified amino acid at the correct point for addition to the growing peptide chain. The inability of the aminoacyl synthetase to catalyze the attachment of the amino acid would leave the corresponding tRNA unchanged and capable of exerting its postulated repression of RNA synthesis, while protein synthesis would halt as soon as a codon sequence appears in the mRNA for which no corresponding tRNA is available. Why are up to 6 enzymes out of the 18 assayed affected? Are these enzymes related genetically, or is there some degree of cross-function between them? Berg et al. (1961) reported that isoleucyl-tRNA synthetase catalyze not only the isoleucyl-AMP enzyme complex, but also a valyl-AMP enzyme complex, depending upon the concentration of tRNA.

Considering the second part of the sequence, each aminoacyl RNA molecule of a given species requires a unique structure. The unusual primary structure of yeast alanyl-tRNA (Holley et al. 1965) offers strong inference that this may be the case while Sarin et al. (1966), using the technique of optical rotatory dispersion to study conformational changes, presented data indicating a difference in

the secondary (and tertiary) structures of several purified yeast tRNA's. The tRNA's underwent conformational changes between 22°C and 40°C, consonant with their individualistic bioligical roles in the esterification of amino acids. Therefore, rather than enzyme inactivation, alteration of the site of attachment on the tRNA molecule for the enzyme might be involved or, the tRNA might alter the specificity of the aminoacyl synthetase by changing its conformation (allosteric alteration) as has been reported by Loftfield and Eigner (1965).

The last study on the effect of rate of RNA synthesis on subsequent protein synthesis points to the importance of the one to the other. The presence of accumulated RNA, whether of all three classes or not is not known, in cells exposed to 30°C, permits protein synthesis to continue. The death rate of cells at 30°C has been shown to be slower the more nutrients present in the medium. RNA concentration of a cell in balanced growth is proportional to the growth rate a particular medium is capable of supporting, and where the death rate is slowest, RNA concentration prior to transfer has been highest, and vice versa. Reasons must be put forward to explain why the RNA synthesized at 30°C, although rate of synthesis is increased with temperature for some time, is unable to sustain protein synthesis. Again, factors causing minor modification to the structure and hence functional capacity of certain species of RNA would seem to be the most plausible explanation.

If chemical modification of the RNA were to occur, say because of an activated degradative enzyme, this effect might be lessened if enough RNA were synthesized to more than saturate the enzyme. This brings to mind the evidence suggesting the presence of an active phosphodiesterase at 30°C, namely the release of huge amounts of 5'-nucleotides of the RNA bases from the cell.

Transfer RNA would be particularly susceptible to its action. The considerable advance made by Holley et al. (1965) in describing the complete primary sequence of an alanyl-tRNA from yeast confirmed earlier suggestions that the nucleotide sequence of such a molecule permits formation of a structure with several loops connected by double-stranded regions. The amino acid-linking end of all species of tRNA consists of a cytidylate-cytidylate-adenylate (-pCpCpA) sequence with a 3'-hydroxyl terminal. RNA nucleotides complexed in a hydrogen-bonded double-helical structure are strongly protected against enzymatic degradation (Geiduschek, Moohr and Weiss, 1962; Cantoni et al. 1962). The terminal -pCpCpA sequence of tRNA is not protected by such bonding and the 3'-hydroxyl group would favour exonucleolytic attack by a phosphodiesterase which would release the nucleoside A and the 5'-nucleotides, pC. Such activity against tRNA has been demonstrated by Gerg and Ofengand (1958), Nihei and Cantoni (1963) and Zubay and Takanami (1964) using snake venom phosphodiesterase, an enzyme with such a mode of attack; tRNA loses all ability to accept activated amino

acids after less than 5% degradation by this enzyme.

The work being carried out in these areas of cellular biochemistry suggests several lines of interesting, and perhaps fruitful, investigation; and, because of the nature and possible significance of the molecules concerned, any findings may have relevance beyond their relation to the question of obligate psychrophily.

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